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VOL. 47 MARCH, 1956 NO. 3

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Clinical and Experimental

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The Journal of Laboratory and Clinical Medicine

VOL. 47 MARCH, 1956 NO. 3

CLINICAL AND EXPERIMENTAL

SOME ASPECTS OF KIDNEY FUNCTION IN HEPATOLENTICULAR DEGENERATION (WILSON'S DISEASE)

R. E. HODGES, M.D., AND W. M. KIRKENDALL, M.D., IOWA CITY, IOWA, AND C. J. GUBLER, PH.D., SALT LAKE CITY, UTAH

INTRODUCTION

WILSON,[1] in his superb monograph of 1912, postulated that hepatolenticular degeneration might be caused by a toxic agent. Today, forty-three years later, this theory is still considered possible,[2] but the controversy has grown more active in the light of recent discoveries including abnormalities of copper and amino acid metabolism,[3] low plasma levels of ceruloplasmin,[4] and the demonstration of an abnormal distribution of copper in the plasma of patients with hepatolenticular degeneration.[5]

The abnormal deposition of copper in the tissues of patients with Wilson's disease is well documented. The cause of this strange process, whether related to chelation with incomplete proteins,[6] the toxic effects of copper itself,[3] or other factors, is uncertain.

Little is known about the role of the kidney in this complex disease. While it is certain that amino-aciduria and excessive excretion of copper are common features of this disease, there is no record of histologic abnormalities of the kidney.

From the Departments of Medicine, College of Medicine, State University of Iowa, and Veterans Hospital, Iowa City, Iowa, and College of Medicine, University of Utah, Salt Lake City, Utah.

This investigation was supported in part by research grants from the American Heart Association, the Iowa Heart Association, and the Iowa State Department of Health.

Presented in part at the 46th Annual Meeting of the Society for Clinical Investigation, Atlantic City, New Jersey, May 3, 1954.

Received for publication Sept. 19, 1955.

METHODS

To explore the renal defect in patients with Wilson's disease, two types of renal clearance studies were performed. Standard methods for estimating renal plasma flow and glomerular filtration rate were employed, using sodium para-aminohippurate (PAH) and inulin[7] as test materials. Saline was employed as the infusion fluid initially. Subsequently, casein hydrolysate (Amigen) was substituted for the saline. All samples of blood and urine were assayed for alpha amino acid nitrogen[8, 9] and for copper.[10, 11] These studies were repeated several times in three normal medical students and in four patients with Wilson's disease.

Patients were studied in the recumbent position in the morning after discontinuing all medications for twenty-four hours. Fifteen hundred milliliters of water was given by mouth one to two hours before the test. Urine flows of 4 to 8 ml. per minute were maintained by giving 200 ml. of supplementary water to drink each hour. The priming injection of PAH and inulin was given and followed with an infusion of these two substances initially in 0.9 per cent saline and subsequently in 5 per cent casein hydrolysate solution. In all instances, plasma levels of PAH and inulin were maintained at a reasonably constant level (between 1.5 and 4.0 mg. per 100 ml., and 25 and 50 mg. per 100 ml., respectively).

After equilibration for 30 to 40 minutes, urine collections were made for four 14-minute clearance periods. Blood was collected one minute prior to the mid-point of the period through an indwelling Unger needle in an arm vein. All urine was collected through an indwelling catheter, and the bladder was rinsed at the end of each period with saline and air.

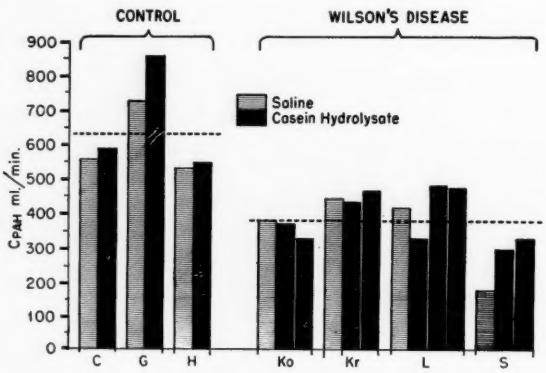

Fig. 1.—The renal plasma flow in three normal persons and in four persons with Wilson's disease, with saline and casein hydrolysate as the infused solutions.

RESULTS

Renal extraction of PAH was determined in one patient (M.L.), blood being drawn simultaneously from the right renal vein and an antecubital vein on four separate occasions over a 2-hour period. Nitrogen balance studies were done in the four patients with Wilson's disease over a 10-day period, and they were found to be in a positive nitrogen balance.

The patients with Wilson's disease showed a consistent pattern of reduced renal plasma flow (Fig. 1) as compared with the normal subjects. Similarly, glomerular filtration rate (Fig. 2) was reduced in these patients. Although infusion of casein hydrolysate had no appreciable effect upon renal plasma flow, it did give false elevation of glomerular filtration rate (Fig. 2), due to casein hydrolysate interference with determination of inulin in blood samples. Clearance rates of amino acid nitrogen were initially greater in two of three patients

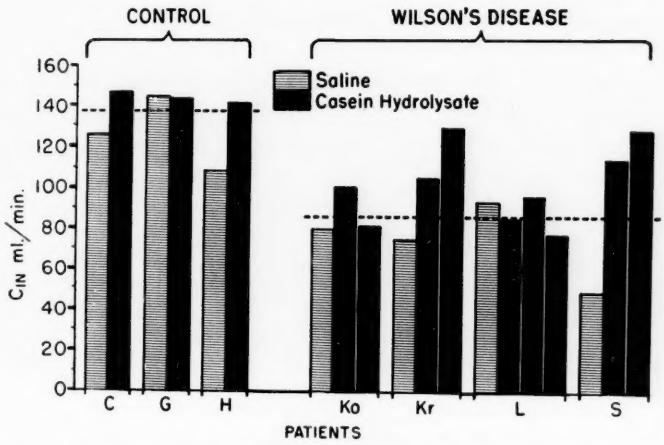

Fig. 2.—The glomerular filtration rate of three normal persons and four persons with Wilson's disease, with saline and casein hydrolysate as the infused solutions.

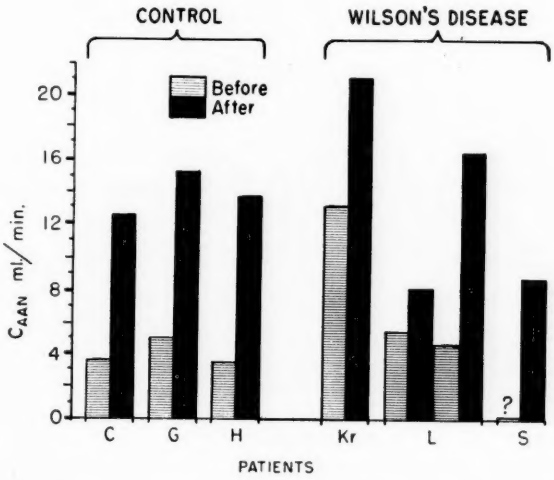

Fig. 3.—The renal clearance of amino acid nitrogen before and after the infusion of casein hydrolysate in three normal persons and four persons with Wilson's disease.

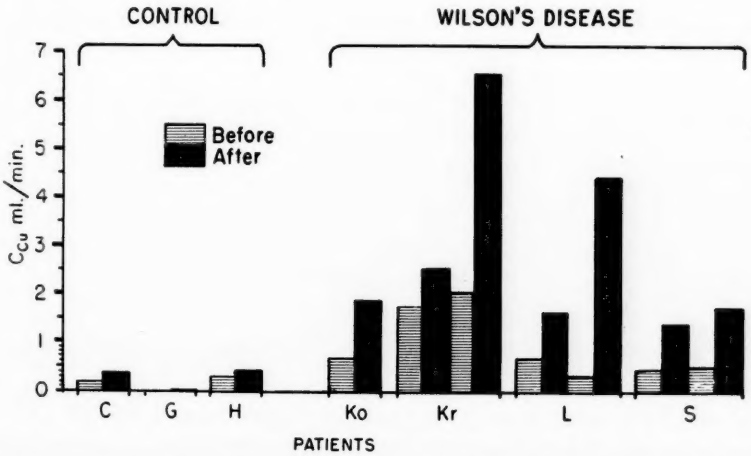

Fig. 4.—The renal clearance of copper before and after casein hydrolysate in three normal persons and four persons with Wilson's disease.

with Wilson's disease than in normal persons (Fig. 3), but following infusion of casein hydrolysate, the degree of rise in amino acid nitrogen clearance was similar in both groups. Although our data on this point are meager, the findings are in close agreement with those of Uzman and Denny-Brown[14] and Cooper and co-workers.[12] At the same time, however, clearance rates of copper (Fig. 4) promptly rose in the patients with Wilson's disease but remained unchanged in the medical students. A comparison between clearance rates of copper and amino acid nitrogen shows close parallelism (Fig. 5).

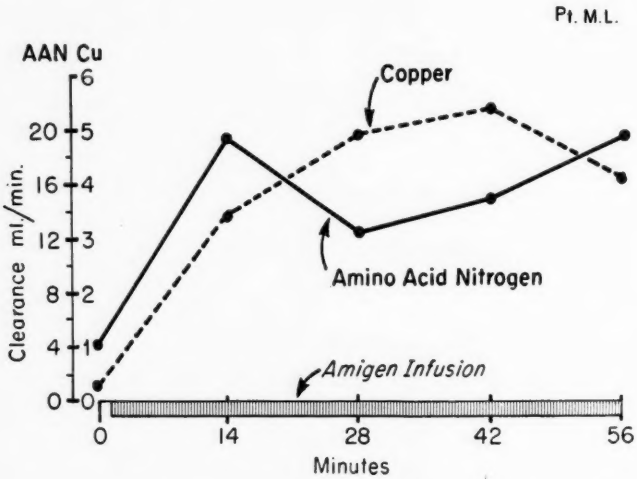

Fig. 5.—The relationship between copper and amino acid nitrogen renal clearances in a patient with Wilson's disease.

DISCUSSION

Earlier studies by others[12] showed that patients with Wilson's disease excreted increased amounts of copper, of amino acids, and occasionally of glucose in the urine. The finding that renal plasma flow was reduced to a greater extent than glomerular filtration rate suggests abnormal vascular resistance in the renal circulation.

The clearance of amino acid nitrogen in these patients during infusion of casein hydrolysate rises to the same degree as in normal subjects, but it is greater than normal during a fasting state when only saline is infused. These findings are almost identical to those of Cooper and associates.[12] This situation might be explained by a defect in tubular reabsorption of amino acid nitrogen in patients with Wilson's disease. Thus, during an infusion of a mixture of amino acids the tubular reabsorptive maximum (Tm_{AA}) for some of them may be exceeded in normal subjects, so that excretion of amino acid nitrogen approaches the glomerular filtration rate as the blood concentration rises. Hence, the excretion would become nearly the same as that found in patients with Wilson's disease whose Tm_{AA} may be lower. The rate of copper clearance in

these patients varies directly with the rate of amino acid clearance. This supports Uzman's[13] hypothesis of competitive tubular reabsorption of free amino acids and oligopeptides.

The data reported in this study suggest that the renal defect involves primarily the renal tubules. Tubular excretory mechanisms for PAH are probably intact since the PAH extraction in one of the four patients was 92 per cent. Renal glycosuria,[12] as well as amino-aciduria, may occur in some patients with Wilson's disease. This defect may be comparable to that found in the de Toni-Fanconi syndrome in which hepatic cirrhosis may develop.[2]

The absence of clinical derangements of acid-base balance and fluid regulation in patients with Wilson's disease implies that distal tubular function is intact. The occurrence of elevated filtration fraction and total renal vascular resistance probably indicates that arteriolar spasm is present. However, none of the four Wilson's disease patients demonstrated arterial hypertension or retinal vascular changes. Pathologic sections of kidneys from patients with Wilson's disease are routinely reported as appearing normal.[1] An examination by one of us (W. M. K.) of hematoxylin- and eosin-stained kidney sections from patients with Wilson's disease at the Armed Forces Institute of Pathology revealed no consistent abnormalities.* More important is the occasional finding of a combination of abnormalities of tubular transfer for glucose and amino acids, since both are reabsorbed in the proximal tubule.

SUMMARY AND CONCLUSIONS

Four patients with Wilson's disease were studied for renal abnormalities. Decreased renal plasma flow and glomerular filtration rate were found with a high filtration fraction. The patients were in a positive nitrogen balance despite amino-aciduria. The degree of amino-aciduria after casein hydrolysate infusion paralleled the degree of copper excretion. Plasma clearance of copper was high in patients with Wilson's disease after both saline and protein hydrolysate infusion. The evidence suggests that the functional renal defect is confined to the proximal convoluted tubule and the renal vascular tree.

We are indebted to Drs. William B. Bean, George E. Cartwright, Charles Schwartz, and John B. Wild for their aid and constructive criticism.

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*Brigadier General Raymond Dart, MC, USA, Commandant, Armed Forces Institute of Pathology, gave permission to examine this material.

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THE BLOOD AMMONIA IN NORMAL PERSONS

W. Graham Calkins, M.D.
Kansas City, Kan.

IN THE past five years, there has been increased interest in the blood ammonia concentration in hepatic disease. Numerous papers reporting both normal and abnormal blood ammonia concentrations have appeared in the recent literature. There has been much speculation as to whether the normal blood in vivo contains ammonia or not. The current trend of thought is that the circulating blood contains no ammonia but that ammonia is formed rapidly after the blood is shed. It was the purpose of the present study to investigate this concept and to establish values for the normal blood ammonia. The term "blood ammonia" is used in this report with the understanding that it refers to the ammonium ion nitrogen present in the blood.

MATERIALS AND METHODS

Serial blood ammonia determinations were done at intervals of 10, 20, 30, 60, 120, and 180 seconds after blood was shed in ten patients without liver disease chosen at random. An indwelling needle, which had previously been autoclaved and kept free from ammonium contamination was placed in the patient's vein. Blood was drawn through this needle into tuberculin syringes, each containing a small amount of ammonium-free potassium oxalate. The blood was then introduced directly from the tuberculin syringes into the Conway dishes at the desired time after shedding.

The actual determination of the blood ammonia concentration was carried out by a modification of Conway's method.[1] The inner chamber of a Conway dish was filled with 1 ml. of 0.0002 N hydrochloric acid containing methyl red–methylene blue indicator. The outer chamber contained 1 ml. of ammonia-free saturated solution of potassium carbonate. One milliliter of oxalated blood was introduced rapidly into the outer chamber directly from the tuberculin syringe that it was drawn in. The cover which had been previously coated with a mixture of mineral oil and paraffin was placed on the Conway dish, and the unit was rotated twenty times to ensure adequate mixing of the blood and potassium carbonate. Diffusion was allowed to proceed for fifteen minutes at room temperature. Then the hydrochloric acid in the inner chamber was titrated with 0.0005 N barium hydroxide. A blank and a standard (ammonium sulfate–100 μg of ammonia nitrogen per 100 ml.) were run simultaneously with the blood. No correction was made for the slight difference in the rates of diffusion of ammonia from aqueous and sanguineous solutions. Results were expressed as micrograms of ammonia nitrogen per 100 ml. of blood.

With use of the above modification of Conway's technique for the determination of blood ammonia, the error on standard ammonium sulfate solutions was 9 per cent or less. The error, when the ammonia concentration of the same blood sample was determined repeatedly was 13 per cent or less. Measured amounts of ammonium nitrogen were added

From the Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kan.
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to whole blood and the resulting concentration of ammonia measured. On these recovery experiments there was an error of 10 per cent or less between the predicted and the actual resulting ammonia concentrations.

In five additional patients, blood was drawn with use of standard venipuncture technique and introduced into a test tube containing dried ammonia-free potassium oxalate. The blood ammonia concentration was determined at intervals of 3, 5, 10, 20, 30, 40, 50, and 60 minutes after shedding. In addition, the blood ammonia concentration was determined in 100 normal persons who were accepted by the blood bank as donors. In all cases, the donor's blood was introduced into the Conway unit within five minutes after it was shed. There were 87 males and 13 females, whose ages ranged from 18 to 53 years, with an average of 31.2 years. In each case the donor had been fasting for at least four hours prior to determination of the ammonia concentration of his blood.

RESULTS

In each of the ten patients in whom the blood ammonia concentration was determined from 10 to 180 seconds after the blood was shed, there was no significant difference between the values obtained at 10 seconds and the values obtained at 180 seconds after shedding. The average ammonia concentrations 10, 20, 30, 60, 120, and 180 seconds after the blood was shed were 65, 68, 73, 68, 70, and 73 μg per 100 ml., respectively (Table I). Fig. 1 shows the concentration of blood ammonia from 10 to 180 seconds after shedding in one patient. The variations in the values are within the error of the method and indicates that there is no significant change in the blood ammonia concentration between 10 and 180 seconds after shedding.

TABLE I. BLOOD AMMONIA NITROGEN CONCENTRATIONS AT VARYING INTERVALS AFTER BLOOD WAS SHED IN TEN PATIENTS (μG PER 100 ML.)

PATIENT	SECONDS AFTER BLOODSHED					
	10	20	30	60	120	180
1	69	78	--	75	75	79
2	75	93	89	87	90	99
3	90	82	75	78	77	82
4	44	59	59	46	64	55
5	81	65	74	78	80	88
6	60	76	84	80	79	81
7	65	69	76	79	73	79
8	51	38	--	45	45	40
9	58	65	67	62	63	73
10	58	59	62	47	54	54
Average	65	68	73	68	70	73

TABLE II. BLOOD AMMONIA NITROGEN CONCENTRATIONS AT VARYING INTERVALS AFTER BLOOD WAS SHED IN FIVE PATIENTS (μG PER 100 ML.)

PATIENT	MINUTES AFTER BLOODSHED							
	3	5	10	20	30	40	50	60
1	--	78	60	86	129	158	192	219
2	47	--	68	58	69	106	116	124
3	68	76	86	111	120	152	177	204
4	67	--	73	72	90	--	116	130
5	85	85	95	106	133	185	207	260
Average	65	79	76	87	108	150	162	189

In each of the five patients in whom the blood ammonia was determined from three to sixty minutes after shedding, there was a gradual rise in the

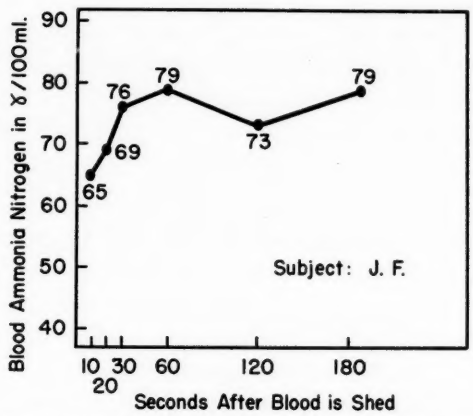

Fig. 1.—Curve showing relatively little change in the ammonia concentrations in blood from 10 to 180 seconds after shedding.

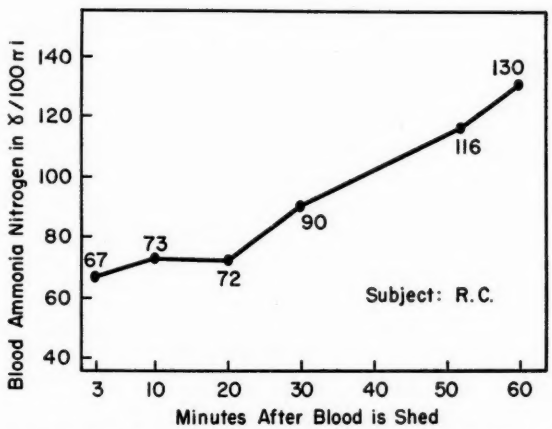

Fig. 2.—Curve showing blood ammonia concentrations from 3 to 60 minutes after shedding.

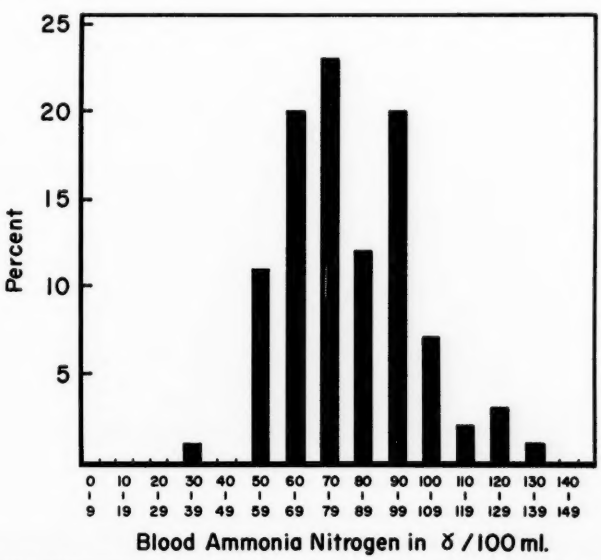

Fig 3.—Percentile distribution of various blood ammonia concentrations in 100 normal persons.

ammonia concentration, commencing about twenty minutes after shedding of the blood. The average blood ammonia concentrations 3, 5, 10, 20, 30, 40, 50, and 60 minutes after shedding were as follows: 67, 79, 76, 87, 108, 150, 162, and 189 μg per 100 ml., respectively (Table II). Fig. 2 shows the concentration of ammonia from three to sixty minutes after blood was shed in one patient. By the combination of the results of these fifteen patients it can be seen that there is no appreciable change in the blood ammonia concentration between ten seconds and twenty minutes after shedding.

The ammonia concentration of the 100 normal blood donors ranged from 34 to 133 μg with a mean of 79.5 (standard deviation 2.68) μg per 100 ml. Ninety-three per cent of the donors had blood ammonia concentrations ranging from 50 to 110 μg per 100 ml. Fig. 3 shows the percentile distribution of the blood ammonia concentration in 100 normal persons.

DISCUSSION

The results of the present study indicate that there is no significant change in the blood ammonia concentration from ten seconds to twenty minutes after the blood was shed. Therefore, it is postulated that there actually is a small amount of ammonia in the blood in vivo. This is in disagreement with Conway.[4] By a microdiffusion method he determined the concentration of ammonia in the blood of 159 normal persons at various times after their blood was shed. He found that there was a steep rise in the blood ammonia up to three minutes after the blood was shed, followed by a slower gradual increase in its concentration. In his work, he determined the blood ammonia as early as forty-five seconds after shedding. By extrapolation of the curve formed by the values of the blood ammonia at various times after shedding, Conway concluded that there was no free ammonia in the circulating blood. He applied the term "alpha ammonia" to the ammonia that was formed in the first few minutes after the blood was shed. By drawing the blood in an atmosphere of pure carbon dioxide, he was able to inhibit the formation of the "alpha ammonia." Blood drawn under such conditions formed no ammonia up to 128 minutes after shedding.

In 1937, 1938, and 1939, Conway and Cooke[2, 3, 4] demonstrated that there were three phases of ammonia formation in shed blood. The "alpha ammonia," amounting to about forty μg per 100 ml., formed immediately after shedding and was thought to be derived from the breakdown of adenosine. The "beta ammonia" was formed within three to five hours after the blood was shed. Its concentration was approximately 1 mg. per 100 ml. and was thought to be due to the breakdown of adenyl-pyrophosphate. Later after shedding, the "gamma ammonia" was formed and this was thought to be derived from the deamination of vegetable adenylic acid.

In 1947 Kalckar[5] isolated an enzyme that deaminated adenosine, but he was unable to demonstrate any inhibitory effect of carbon dioxide on its

action. This would seem to contradict Conway's report that the deamination of adenosine in the blood depended upon the loss of carbon dioxide tension after shedding.

In 1955 White and associates,[6] using a modification of Conway's method for the determination of ammonia, confirmed his earlier work by demonstrating a rapid rise in the concentration of the blood ammonia within a few minutes after the blood was shed. Again, by extrapolation of the curve obtained from the blood ammonia concentrations at various time intervals after shedding, they postulated that the circulating blood ammonia concentration was zero. However, they were unable to inhibit the formation of ammonia by the use of carbon dioxide as Conway had done. Zittle[7] had found that adenosine deaminase was easily inhibited by the silver ion. White and his group were unable to suppress the liberation of ammonia by the use of silver nitrate. This seemingly refutes Conway's idea that the "alpha ammonia" is formed from the deamination of adenosine. Other investigators[8, 9] have drawn blood in an atmosphere of carbon dioxide and have failed to inhibit the formation of high ammonia concentrations.

The ammonia level found from ten seconds to twenty minutes after the blood was shed corresponds to the "alpha ammonia" of Conway and Cooke.[2, 3, 4] If there were no ammonia in the blood in vivo, then one must postulate that the "alpha ammonia" was formed during the ten seconds that the blood was being drawn and introduced into the Conway dish. It is doubtful that this could occur, since the blood was in the presence of potassium oxalate which should inhibit any enzymatic action. Moreover, it is highly improbable that the blood adenosine would be completely deaminated in ten seconds and if it were only partially deaminated, we should expect to find an increasing ammonia concentration of the blood due to further deamination of adenosine as the time after shedding increases.

About twenty minutes after shedding, there was noted the onset of a steady increase in the blood ammonia. This was assumed to represent the "beta ammonia" reported by Conway. Accordingly, it was felt that blood ammonia concentrations could be accurately determined up to twenty minutes after shedding. For all practical purposes, it makes no difference whether the ammonia concentration is determined one or ten minutes after the blood is shed. It remains constant up to twenty minutes after shedding. Probably in extremely warm weather, this time limit would have to be reduced further due to more rapid decomposition of the blood.

Since 93 per cent of the normal persons had blood ammonia concentrations ranging from 50 to 110 μg per 100 ml., these were accepted as normal values. Concentrations above 110 and below 135 μg per 100 ml. are in the borderline zone and cannot be considered definitely normal or abnormal. Values above 135 μg per 100 ml. are definitely abnormal.

It is to be emphasized that the blood ammonia was determined ten seconds after shedding in only ten patients. This is a relatively small number, as compared to Conway's series of 159 patients; but in each patient in our

series there was a relatively large ammonia concentration in the blood ten seconds after shedding. It is recognized that the error of the method used for the determination of the blood ammonium concentration in this study is relatively large as compared to that of other quantitative biochemical determinations. However, it is felt that the error is not large enough to change the significance of the values reported in this study. It seems justified to conclude that there is a significant amount of ammonia in the blood in vivo.

SUMMARY

1. Serial determinations of the blood ammonia in fifteen patients showed no appreciable change from ten seconds to twenty minutes after shedding of the blood. It is postulated that there is a significant amount of ammonia in the circulating blood.

2. Fasting blood ammonia concentrations in 100 normal persons ranged from 34 to 133 μg per 100 ml., with an average of 79.5 μg per 100 ml. (standard deviation 2.68).

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RELATION OF COMPLEMENT FIXATION TO RENAL DISEASE IN RATS INJECTED WITH DUCK ANTIKIDNEY SERUM

ABRAM B. STAVITSKY, PH.D., V.M.D.,* WALTER HEYMANN, M.D., AND DONALD B. HACKEL, M.D.
CLEVELAND, OHIO

IN A previous study[1] serum complement was shown to be markedly reduced within 30 minutes following intravenous injection of rabbit antirat kidney serum into normal or bilaterally nephrectomized rats. Complement was similarly decreased in rats given this antiserum after absorption of antibodies to rat plasma and rat erythrocytes. Complement may, therefore, be fixed in vivo as a consequence of extrarenal tissue antigen-antibody reactions. However, the relationship of complement fixation to the development of renal disease was not elucidated in this study.

The present investigation was based on suggestions of a different pathogenetic mechanism for the renal disease in rats resulting from injection of *duck* antikidney serum compared to that following inoculation of *rabbit* antikidney serum. Kay,[2] Heymann and co-workers,[3] and Hasson and Seegal[4] observed that injection of *duck* antikidney serum into rabbits or rats was followed by a latent period of 3 to 20 days before the onset of kidney disease, whereas this disease developed without latent period after inoculation of *rabbit* antikidney serum. Kay explained the lag with duck antiserum as follows: first the antibodies combined with kidney antigen in vivo without damage to the organ. Then the recipient produced antibodies against the uncombined duck serum proteins. Finally these antibodies reacted with the duck antibodies initially localized in the kidney; this combination caused renal damage. The latent period was, therefore, the time required for development of antibodies to duck serum before kidney damage occurred.

Kay has presented strong evidence in support of his hypothesis.[2, 5] Pressman, Korngold, and Heymann[6] showed further that kidney-localizing antibodies were present in duck as well as rabbit antirat kidney sera. The latent period with duck antisera thus cannot be attributed to failure of localization of antibody in kidney. These workers[6] attributed the different response to the rabbit and duck antikidney sera to rapid fixation of complement by the rabbit antibodies and absence of fixation by duck antibodies. Only after the rats produced anti-

From the Departments of Microbiology, Pediatrics, and the Department of Pathology at City Hospital, Western Reserve University School of Medicine, Cleveland, Ohio.

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bodies to the injected duck serum would complement be fixed in the kidney and damage occur. In support of this hypothesis they drew an analogy to the decisive role of complement in immune hemolysis and bacteriolysis and suggested that in vivo complement fixation might also be involved in the kidney damage produced by antikidney sera. They[6] also cited in vitro studies[7] in which rabbit antibodies fixed complement but duck antibodies did not.

The present study was designed to evaluate the role of complement in the pathogenesis of kidney disease following injection of antikidney serum obtained from ducks. Earlier work by Ogawa and Sato[8] suggested a relationship between complement and the development of renal disease. Recent studies by Lange,[9] reported since the conclusion of the present study, found good correlation between in vivo complement fixation and onset of kidney damage in rabbits inoculated with duck antikidney serum.

Two technical features of the present study should be emphasized. First, the bleeding of rats from the infraorbital sinus[10] permitted daily complement determinations and thus minimized the chances of missing any change in this component. Secondly, the combination of frequent bleedings with frequent determinations of chemical changes in urine and blood, blood pressure, titrations of antiduck serum titers, and histologic examinations of the kidney provided opportunity to observe any relationship between complement, renal disease, and antiduck serum titers.

MATERIALS AND METHODS

Animals.—Sprague-Dawley rats of both sexes, weighing 130 to 160 grams, were used.

Preparation of Antisera.—The duck antibodies to rat kidneys were prepared as described previously.[3]

Assay of Complement.—Complement was assayed by the procedure of Ecker, Hiatt, and Barr,[11] in terms of 50 per cent hemolytic units. The number of milliliters of serum required to lyse 50 per cent of a standard suspension of sensitized sheep red blood cells was determined. The *hemolytic titer* of a serum represents the reciprocal of the volume of serum required for 50 per cent lysis. Thus if 0.1 ml. of serum was required, the hemolytic titer would be 10 units.

In 5 separate experiments the rat sera obtained at various times after injection of duck antikidney sera were found to lack anticomplementary properties.

Estimation of Antibody.—Rat antibody to duck serum was estimated by the hemagglutination reaction. This method has recently been described in detail.[12] Normal duck serum diluted 1:2 with saline was used for sensitization of the tannic acid-treated red blood cells.

Other Procedures.—Rats were bled from the infraorbital sinus.[10] About 0.25 ml. of blood was taken each time. The severity of renal disease was determined by quantitative measurements of proteinuria, by the presence of anasarca, changes in blood chemistry (blood-urea nitrogen, serum protein, total lipids, and cholesterol), blood pressure, and histologic changes as described previously.[13] In each experiment the rats were bled and the duck antikidney serum immediately injected intravenously in one dose. The volume of nephrotoxic serum injected was related to the kidney weight of the recipient rat as described previously[13] and fractions or multiples of this dose injected.* Thirty minutes, and at various daily intervals, after injection of the serum, the animals were bled again. The serum was separated, quick-frozen in CO_2-alcohol, and placed in a Deepfreeze at -25° C., until the sera from each experiment were assayed on the same day.

*For instance, the renal dose was 1.4 ml. of antiserum for a 100 gram rat. For dosages given rats of other weights see the table in reference 13.

EXPERIMENT

Correlation Between In Vivo Complement Fixation and Development of Renal Disease.—Forty-three rats were injected intravenously with varying amounts (⅓ to 1½ *renal doses**) of duck antirat kidney sera. Eight different sera derived from various bleedings of four different ducks were used. Nine rats were similarly injected with comparable amounts of normal duck serum. Complement assays were made daily and urinalysis and blood pressure determinations daily or every other day. Upon death or sacrifice of the rats the kidneys were examined for histologic changes.

TABLE I. SUMMARY OF DATA ON RELATION OF COMPLEMENT FIXATION TO RENAL DISEASE IN RATS INJECTED WITH DUCK ANTIRAT KIDNEY SERUM

NO.	TYPE OF RESPONSE		NO. OF RATS	PER CENT OF TOTAL
	DISEASE	COMP. FIX.		
1	−	−	13	30
2	+	−	11	26
3	−	+	11	26
4	+	+	5	12
5	+ before same time	+	3	6

Before—disease preceded complement fixation.
Same time—disease occurred at about same time or shortly after complement fixation.

TABLE II. RELATION OF COMPLEMENT FIXATION TO DEVELOPMENT OF RENAL DISEASE IN RATS INJECTED WITH DUCK ANTIRAT KIDNEY SERUM

TYPE OF RESPONSE*	RAT NO.	FIXATION DAY†	HEMOLYTIC TITER OF SERUM‡			RENAL DISEASE ONSET DAY
			BEFORE	DURING	AFTER FIXATION	
2	87	None				2
	110	None				4
3	69	8	41.7	8.3	62.5	None
	73	2	50.0	12.5		None
	111	7	50.0	12.5	83.3	None
	123	30 min.	22.7	8.9	19.2	None
4	108	6	45.5	10.0		0
	116	7	45.5	25.0	13.9	3
5	68	14	31.0	15.5		15
	99	2	25.0	12.5		3

*Refers to categories in Table I.
†Day on which complement fixation first observed.
‡Before—day before fixation occurred; after—day after fixation occurred.

Table I summarizes the results, and Table II presents data for selected animals exhibiting each type of response. It can be seen that over half (56 per cent) of the rats showed no drop in complement during the two and one-half weeks of observation. Another quarter did not develop disease but did show complement fixation. In 12 per cent, disease preceded complement fixation by several days. In only 3 rats (6 per cent) were complement fixation and the onset of renal disease correlated.

Of particular interest in this series was the development of kidney disease in 4 out of 43 rats without a latent period (see Rat 108 in Table II). Proteinuria

**For footnote see page 350.*

was definite in these animals within 24 hours after injection. However, none of the animals which immediately developed nephrotic renal disease showed a concurrent drop in complement. It was also interesting to note that 5 rats showed complement fixation within 30 minutes after injection of the antikidney serum (see Rat 123 in Table II). None of these animals developed kidney disease for several days.

In 4 out of the 9 rats injected with normal duck serum, complement fixation occurred within 3 to 6 days after injection, whereas in 5 of them no change in complement was observed. Renal disease did not occur in any of them.

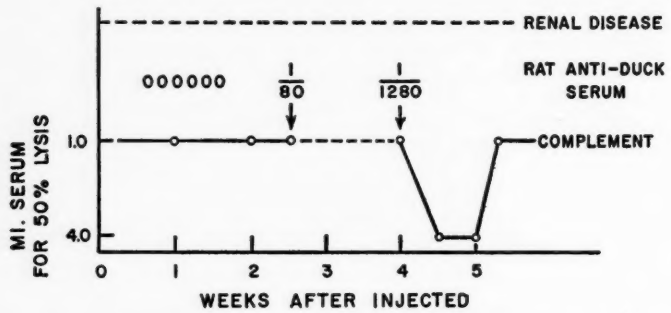

Fig. 1.—Correlation of complement fixation, antiduck serum titers and renal disease in rats injected with duck antikidney serum.

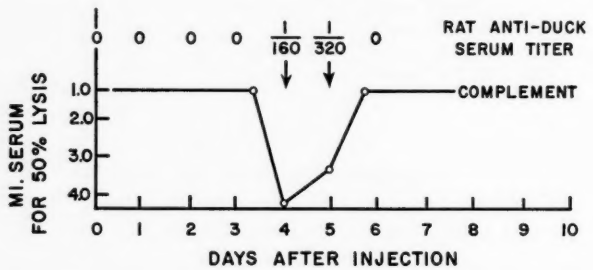

Fig. 2.—Relations of rat antiduck serum titers and complement fixation in a rat injected with normal duck serum.

Correlation Between Time of Appearance of Antibodies to Duck Serum, Complement Fixation, and Kidney Disease.—Complement and antibody titers were determined daily following injection of duck antikidney serum into 10 rats and normal duck serum into 5 rats. In the antiserum-injected animals, evidence of renal disease first appeared within 1 to 2 days after the antibodies to duck serum were detectable. In 8 of them the appearance of antibodies was followed within a day or two by a drop in complement. Of the other 2, complement was fixed six days after the appearance of antibodies in one rat and ten days later in the other. Fig. 1 illustrates the data obtained in one animal. The injection of antiserum was followed by a drop in complement four weeks later, although no disease occurred. At this time, the antiduck serum titer was 1/1,280. However, a week and one-half earlier, when there was no detectable drop in complement, the serum titer was 1/80. No sera were available from the period from 2½ to 4 weeks.

Fig. 2 presents typical data obtained on injection of normal duck serum. In this series 4 out of 5 rats showed good correlation between the appearance in the serum of antibody to duck serum and a drop in complement. The fifth animal neither developed antibodies to duck serum nor showed a decrease in complement.

Incidence of Renal Disease Without Latent Period and Correlation Between Length of Latent Period and Severity of Renal Disease.—Since 4 rats in the first series developed renal disease without a latent period, it was of interest to determine the incidence of disease without a latent period in a larger series of rats. Fig. 3 illustrates the results obtained with a second series of 70 rats.

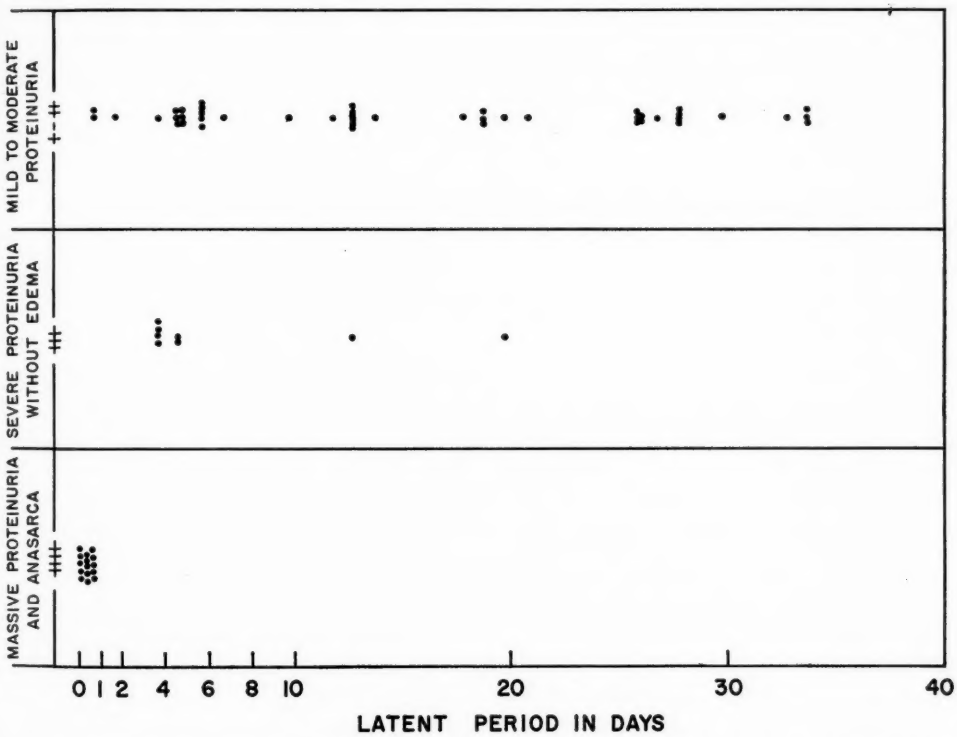

Fig. 3.—Length of latent period in relation to severity of renal disease in 70 rats injected with nephrotoxic serum obtained from ducks.

It can be seen that 15 rats in this series developed kidney disease without delay. The data also show a relationship between the severity of the disease and the length of the latent period. The more severe the renal disease, the shorter was the latent period.

DISCUSSION

These data differ in two respects from those of most previous studies. First, in contrast to the work of Kay[2] in rabbits, from 10 to 20 per cent of the rats in two series developed renal disease *without* a latent period after injection of duck antikidney serum. These results are, however, in agreement with those

reported recently by Hasson and Seegal in rats. Secondly, kidney disease occurred often without obvious relationship to complement fixation.

It is not understood why renal disease developed without a latent period. Seegal[14] presented evidence that the occurrence of this disease without a lag was correlated with the injection of large amounts of kidney antibody. If complement fixation were required for kidney damage and fixation occurred more readily with larger amounts of antibody, damage might occur more readily with greater amounts of antibody. However, in none of the rats which developed disease immediately was complement fixed concurrently. On the other hand, in agreement with Hasson and Seegal, rats which did *not* show a latent period manifested much more marked proteinuria and anasarca than those which did show a latent period. These more marked symptoms were probably associated with the production of greater kidney damage by more potent antisera.

Whatever the explanation, it is obvious that renal disease may develop immediately without the production by the rat of antibodies to duck serum, as postulated by Kay[2] from experiments with rabbits. Occasionally, therefore, injection of *duck* antikidney serum can damage the rat kidney directly with rapid onset of renal disease, such as is seen consistently after injection of *rabbit* antirat kidney serum. However, the length of latent period with duck antisera is related to their potency, whereas one can dilute rabbit sera and still produce disease without a lag.

It is also not understood why the present data disagree with previous data on the relationship of complement fixation to renal disease. The species of animal employed may be important. Ogawa[8] and Lange[9] employed rabbits, and in the latter study, particularly, there was good correlation between the time of fixation and onset of disease. Lange[9] postulated that the difference in results between rat and rabbit might be due to the greater ability of the latter to produce precipitating antibody. In support of his view, only rarely in the present study could a precipitin reaction be obtained with duck serum and rat antibodies to this serum. These antibodies could be detected only by the highly sensitive hemagglutination procedure. On the other hand, Kay[2] consistently obtained precipitin reactions with duck serum and the sera of rabbits which developed antibodies upon injection of duck serum. Since in vitro complement fixation depends on the presence of adequate antibody, in vivo fixation probably has the same requirement.* In fact, it was shown in the present study that small amounts of antibody to some serum constituent could be present without detectable loss of complement (Fig. 1). It is, therefore, conceivable that enough antibody may be present to produce renal disease without obvious complement fixation. Hemolytic complement activity depends on the presence of adequate amounts of each of the four complement components, and fixation might not be detected unless it involved loss of that component present in limiting concentration. Finally, it is possible that a complement drop of short, transient duration may have been missed.

*The requirement of circulating antibody for in vivo complement fixation was previously shown by one of us.[1]

In contrast with the failure of Izumi[7] to obtain in vitro complement fixation with duck antisera was the occasional in vivo fixation with such antisera in the present study (see Rat 123 in Table II). Again species differences may be involved, and duck antisera might fix rat complement better than rabbit complement.

It should be emphasized that the present data do not exclude the participation of complement in cytotoxic phenomena in vivo. In fact, there is a good deal of circumstantial evidence[16, 17, 18] in support of such participation. However, the relationship of complement to these phenomena may be fortuitous rather than one of cause and effect. Tissue antigen-antibody reactions might result in complement fixation and tissue damage by related but independent mechanisms. Thus Ungar[19] recently has presented evidence for activation of tissue proteases by antigen-antibody reactions in the apparent absence of complement. Obviously, further study is required to elucidate the relationship of complement to the production of tissue injury caused by antigen-antibody reactions.

SUMMARY

Following injection of duck antirat kidney serum, from 10 to 20 per cent of two series of rats developed renal disease without a latent period. There was no correlation between the time of complement fixation and onset of renal disease. In the few rats studied there was some correlation between the time of appearance of antibodies to duck serum and onset of disease. There was a relationship between the severity of the disease and the length of the latent period. Possible reasons for these results were discussed.

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SERUM ELECTROPHORETIC STUDIES ON PATIENTS WITH FAMILIAL PRIMARY SYSTEMIC AMYLOIDOSIS

WALTER D. BLOCK, PH.D., JOHN G. RUKAVINA, M.D., AND
ARTHUR C. CURTIS, M.D.
ANN ARBOR, MICH.

A PRELIMINARY report from this laboratory[1] dealt with the serum electrophoretic findings on five related patients with familial primary systemic amyloidosis. In all of these patients, the presence of an atypical peak between the α_2 and β globulin fractions, which we arbitrarily labeled α_2', was demonstrated.

The study of this group has been expanded to include 66 members of the family in an effort to obtain more detailed information concerning the disease. Results of the clinical investigations, including neurologic examinations, liver function tests, skin biopsies, and bone marrow aspirations, will be reported elsewhere.[2] In an effort to characterize the atypical peak, lipoprotein analyses were carried out[3] with use of the ultracentrifuge technique. Special attention has been paid to the inheritance pattern of the disease in this family.[4]

The present report deals with the complete electrophoretic findings on 66 members of this family.

METHODS

Two milliliters of clear centrifuged serum was diluted with 6 ml. of Veronal buffer at pH 8.6, ionic strength 0.1 μ. The diluted serum was dialyzed for 18 hours against 500 ml. of buffer at 0° C. Patterns were run in the Perkin-Elmer electrophoresis apparatus (Model 238). Experiments were run for one hour at 1.5° C.

Mucoprotein electrophoretic patterns were determined in acetate buffer at pH 4.5, according to the method described by Mehl and associates.[5]

The procedure for quantitation of the protein components involved the usual steps of enlargement of the patterns, tracing by hand, and planimetry. The method is essentially that described by Tiselius and Kabat.[6] The total protein content of the original serum was determined by the biuret method.[7, 8]

Mobility calculations were carried out according to the method described by Longsworth.[9]

RESULTS

Of the 66 subjects studied, 29 showed an atypical protein pattern in the α_2 globulin region. In 15 patients, the α_2 region was divided into two clearly distinguishable peaks. In 14 additional patients, the patterns were characterized by poor resolution in the α_2 area. Thirty-seven gave normal electrophoretic patterns. Representative electrophoretic patterns on these three groups are

From the Department of Dermatology and Syphilology, Medical School and the Institute of Industrial Health, University of Michigan, Ann Arbor, Mich.
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TABLE I. SERUM PROTEIN PATTERNS IN PATIENTS

	NUMBER	SEX, AGE	SYMPTOMS	CLINICAL SIGNS	TOTAL PROTEIN	ALBUMIN GM./100 ML.	ALBUMIN PER CENT TOTAL PROTEIN	α_1-GLOBULIN GM./100 ML.	α_1-GLOBULIN PER CENT TOTAL PROTEIN	α_2-GLOBULIN GM./100 ML.	α_2-GLOBULIN PER CENT TOTAL PROTEIN
Atypical Patterns	1	F, 58	+	+	6.82	4.09	60.0	0.29	4.2	0.61	8.9
	2	F, 33	+	+	6.75	4.17	61.8	0.26	3.9	0.41	6.1
	3	F, 30	–	+	7.00	4.78	68.3	0.24	3.4	0.44	6.3
	4	M, 5	–	–	6.81	4.16	61.1	0.28	4.1	0.37	5.4
	5	M, 3	–	–	6.83	4.24	62.1	0.29	4.2	0.43	6.3
	6	M, 52	+	+	7.17	3.97	55.3	0.34	4.7	0.34	4.7
	7	F, 22	+	+	7.62	4.34	57.0	0.39	5.1	0.50	6.6
	8	M, 32	+	+	8.50	4.24	49.9	0.36	4.2	0.54	6.4
	9	M, 9	–	–	7.02	4.21	60.0	0.32	4.5	0.55	7.9
	10	M, 6	–	–	7.40	4.28	57.8	0.31	4.2	0.67	9.0
	11	M, 52	+	+	7.05	4.03	57.2	0.30	4.3	0.27	3.9
	12	M, 22	–	+	7.12	4.34	61.0	0.28	4.0	0.22	3.1
	13	F, 17	–	–	7.39	4.27	57.8	0.43	5.8	0.20	2.7
	14	F, 24	–	–	8.63	5.05	58.5	0.41	4.8	0.71	8.2
	15	F, 47	+	+	7.17	3.79	52.8	0.44	6.1	0.51	7.1
	16	M, 21	–	–	6.83	3.75	54.9	0.31	4.6	0.81	11.9
	17	F, 48	–	–	8.09	4.86	60.1	0.23	2.9	0.44	5.5
	18	F, 18	–	–	6.50	3.85	59.3	0.27	4.1	0.73	11.2
	19	M, 12	–	–	6.50	3.98	61.3	0.26	4.0	0.67	10.3
	20	F, 69	–	–	7.12	3.76	53.5	0.27	3.8	0.37	5.2
	21	M, 46	–	–	6.60	4.00	60.6	0.24	3.6	0.50	7.5
	22	M, 46	+	+	7.02	4.20	59.9	0.32	4.5	0.59	8.4
	23	F, 17	–	–	7.62	4.48	58.8	0.42	5.5	0.71	9.3
	24	M, 16	–	–	9.47	6.06	64.0	0.61	6.4	0.94	10.0
	25	F, 13	–	–	7.50	4.29	57.2	0.37	4.9	0.78	10.4
	26	F, 12	–	–	7.03	4.24	60.3	0.32	4.5	0.65	9.3
	27	M, 73	+	+	7.62	4.70	61.7	0.24	3.1	0.20	2.7
	28	M, 39	+	+	6.96	4.34	62.3	0.27	3.9	0.56	8.0
	29	M, 78	+	+	7.07	3.84	54.3	0.28	4.0	0.67	9.6
Normal Patterns	30	F, 33	–	–	6.84	4.31	63.0	0.26	3.8	0.47	6.9
	31	M, 26	–	–	7.03	4.28	60.9	0.30	4.2	0.53	7.5
	32	F, 3	–	–	7.03	4.61	65.6	0.32	4.5	0.68	9.7
	33	M, 42	–	–	6.84	4.13	60.4	0.28	4.1	0.60	8.7
	34	F, 53	–	–	6.88	4.03	58.6	0.32	4.7	0.67	9.8
	35	F, 34	–	–	7.61	4.65	61.1	0.27	3.6	0.75	9.8

presented in Figs. 1*A*, 1*B*, and 1*C*. Fig. 1*A* shows the atypical peak in the α_2 globulin region, Fig. 1*B* shows a poorly resolved α_2 area, and Fig. 1*C* shows a normal pattern.

Quantitative results on these electrophoretic patterns are recorded in Table I. The table includes results on the 15 patterns showing the atypical peak, on the 14 poorly resolved patterns, and on 6 normal patterns.

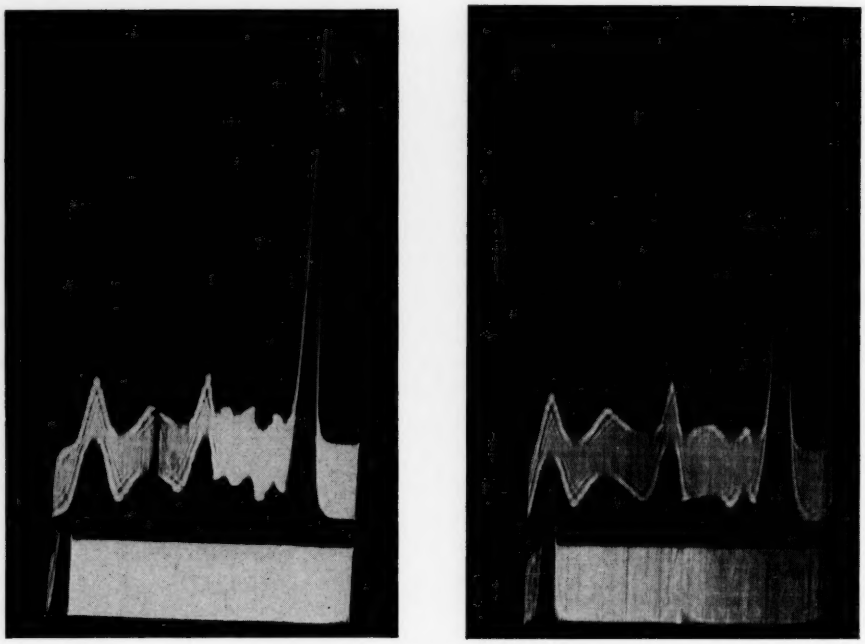
It should be emphasized that the results on the 6 normal subjects recorded in Table I are merely a representative sampling to show the range of normal values obtained. Normal subjects studied included 37 members of the family group and a somewhat smaller number of unrelated individuals. The normal values listed in Table I agree favorably with values reported in the literature.[10, 11]

WITH FAMILIAL PRIMARY SYSTEMIC AMYLOIDOSIS

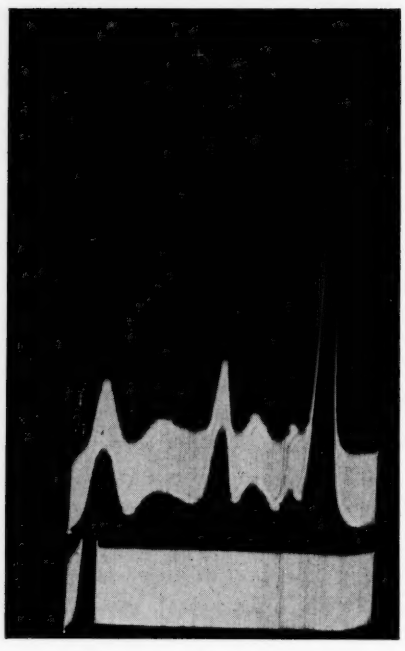
α_2-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		MOBILITIES, $\mu \times 10^5$ CM.^{2} SEC.$^{-1}$ VOLT^{-1}					
GM./100 ML.	PER CENT TOTAL PROTEIN	GM./100 ML.	PER CENT TOTAL PROTEIN	GM./100 ML.	PER CENT TOTAL PROTEIN	ALB.	α_1	α_2	α_2'	β	γ
8.9	—	—	1.02	15.0	0.81	11.9	6.0	5.2	4.3	—	3.3	1.9
6.1	0.20	3.0	0.66	9.8	1.02	15.1	5.8	4.9	4.0	3.4	2.8	1.3
6.3	—	—	0.74	10.6	0.80	11.4	5.7	4.8	3.9	—	2.7	1.2
5.4	0.35	5.2	0.96	14.1	0.69	10.1	5.9	5.0	4.4	4.0	3.0	1.6
6.3	0.44	6.4	0.89	13.1	0.54	7.9	5.8	4.9	4.2	3.8	3.0	1.5
4.7	0.38	5.3	1.13	15.7	1.03	14.3	5.4	4.5	3.8	3.3	2.7	1.5
6.6	0.41	5.4	1.07	14.0	0.91	11.9	5.4	4.6	3.9	3.4	2.7	1.5
6.4	0.65	7.7	1.21	14.2	1.50	17.6	5.9	5.1	4.2	3.7	3.0	1.5
7.9	0.34	4.8	0.91	13.0	0.69	9.8	5.7	4.8	4.1	3.7	2.8	1.5
9.0	0.30	4.0	0.93	12.6	0.92	12.4	5.4	4.7	4.1	3.7	3.0	1.7
3.9	0.54	7.7	0.92	13.0	0.98	13.9	5.7	4.9	3.9	3.6	2.9	1.6
3.1	0.39	5.5	0.69	9.7	1.19	16.7	5.8	5.0	4.3	3.8	2.9	1.5
2.7	0.50	6.7	0.95	12.9	1.04	14.1	5.7	4.8	4.1	3.7	2.7	1.3
8.2	—	—	1.27	14.7	1.19	13.8	5.8	5.0	4.2	—	3.1	1.8
7.1	0.52	7.2	1.10	15.3	0.82	11.4	5.9	5.0	4.3	3.7	3.3	1.9
11.9	—	—	1.05	15.4	0.90	13.2	5.9	5.1	4.3	—	3.2	1.9
5.5	0.44	5.5	0.97	12.0	1.14	14.1	5.7	4.8	4.0	3.5	2.9	1.4
11.2	—	—	0.75	11.6	0.90	13.9	5.9	5.0	4.0	—	3.0	1.5
10.3	—	—	0.95	14.6	0.64	9.8	5.9	4.9	4.2	—	3.1	1.9
5.2	0.60	8.6	0.91	13.0	1.12	15.9	5.7	4.8	4.1	3.6	2.9	1.5
7.5	—	—	0.86	13.1	1.01	15.3	6.0	5.1	4.1	—	3.1	1.6
8.4	—	—	1.16	16.5	0.76	10.8	5.7	4.9	4.0	—	3.0	1.5
9.3	—	—	1.18	15.5	0.84	11.0	5.6	4.7	3.9	—	2.9	1.6
10.0	—	—	1.25	13.2	0.61	6.4	5.5	4.6	3.7	—	2.7	1.2
10.4	—	—	1.16	15.4	0.91	12.1	5.7	4.8	4.0	—	2.8	1.4
9.3	—	—	0.96	13.7	0.86	12.2	5.4	4.6	3.7	—	2.5	1.1
2.7	0.35	4.6	1.16	15.2	0.97	12.7	5.4	4.7	3.9	3.6	2.8	1.5
8.0	—	—	0.84	12.1	0.95	13.7	5.9	5.0	4.2	—	3.2	1.9
9.6	—	—	1.05	14.8	1.22	17.3	5.5	4.7	3.9	—	2.7	1.2
6.9	—	—	0.86	12.5	0.94	13.8	5.7	4.7	3.9	—	2.8	1.5
7.5	—	—	1.01	14.4	0.91	13.0	5.3	4.4	4.0	—	2.9	1.3
9.7	—	—	0.84	12.0	0.58	8.2	5.7	4.9	4.1	—	2.9	1.5
8.7	—	—	1.03	15.1	0.80	11.7	5.3	4.5	3.7	—	2.6	1.6
9.8	—	—	1.21	17.6	0.65	9.4	5.9	5.1	4.1	—	3.1	1.6
9.8	—	—	1.27	16.7	0.67	8.8	5.7	4.8	4.0	—	2.8	1.6

No striking quantitative abnormalities could be demonstrated in these atypical electrophoretic patterns. The albumin content tends to be somewhat low and, conversely, the total globulin content tends to be somewhat elevated. These differences, while suggestive, are not significant. The percentages of the α_1, β, and γ globulins are normal in these subjects. Of the 15 patients showing the atypical peak, 8 had low values for α_2 globulin, and 7 had normal values. The 14 patients with unresolved α_2 globulin areas showed normal values in 12 patients, with slightly elevated values in two. Here again, the trend is suggestive, but not significant.

Mobility calculations were carried out in an attempt to determine the position of the atypical peak. These data are also recorded in Table I. Inspection of the unresolved patterns would seem to indicate that the atypical peak is



A. *B.*



C.

Fig. 1*A*.—Electrophoretic diagram of serum on a patient with familial primary systemic amyloidosis, showing a split in α^2 globulin fraction.

Fig. 1*B*.—Electrophoretic diagram of serum on a patient with familial primary systemic amyloidosis, showing poorly resolved α^2 globulin area.

Fig. 1*C*.—Electrophoretic diagram of normal human serum.

associated with the α_2 globulin fraction. The normal mobility values for α_2 globulin ranged from 3.7 to 4.1. With use of these values as the normal α_2 range, there are 4 patients in which the atypical peak appears to lie between the α_2 and β globulins, and 3 patients in which it appears to lie between α_1 and α_2. In 8 cases, the mobilities of both components fell within the normal α_2 range. On the basis of these data, therefore, the position of the atypical peak remains in question. If one considers the mobility of the peaks in question relative to the mobility of albumin, however, it would seem likely that the atypical peak does lie between α_2 and β globulins.

An attempt was made to determine whether a part of this atypical peak could be mucoprotein. Electrophoretic patterns were run on two atypical sera at pH 4.5. No abnormality in mucoprotein pattern could be demonstrated.

Also recorded in Table I are data on the existence of any clinical signs or symptoms of primary systemic amyloidosis. Of the 37 subjects with normal electrophoretic patterns, none showed manifestations of the disease. Of the 15 subjects who showed the atypical peak, 8 also showed clinical signs and/or symptoms of the disease. Of the 14 subjects whose electrophoretic patterns were unresolved in the α_2 globulin region, 5 had clinical signs and/or symptoms of the disease. There were 16 subjects showing atypical electrophoretic patterns with no manifestations of the disease. It should be noted that 11 of these 16 patients were less than 18 years of age.

DISCUSSION

The question concerning the position of the atypical electrophoretic peak has not been completely resolved. On the basis of mobility determinations on the five cases previously reported,[1] the atypical peak clearly seemed to lie between the α_2 and β globulin fractions. When one considers the results obtained on all of the atypical patterns, the situation is by no means so well defined. Inspection of the unresolved patterns, however, and consideration of mobilities relative to albumin support the original finding that the atypical peak lies between α_2 and β globulins.

Four possibilities concerning the nature of the atypical protein peak have been considered. The component might conceivably be a mucoprotein or a lipoprotein. It might arise from a split in the α_2 globulin fraction or it might represent a protein of unknown, heterogeneous composition. These possibilities are, of course, not mutually exclusive. For example, the atypical component might be heterogeneous and might at the same time arise from a split in the α_2 component. Part of the heterogeneous fraction could conceivably be mucoprotein, or lipoprotein, or both.

Attempts to demonstrate the existence of a mucoprotein abnormality in 2 patients who showed the atypical peak were unsuccessful. These data certainly do not represent unequivocal evidence ruling out mucoprotein as a factor contributing to the atypical peak. If, as has been reported,[5] mucoprotein is associated with the α_1 globulin fraction, it seems unlikely that mucoprotein could contribute to the atypical peak.

Lipoprotein fractionations on these sera have been carried out[3] with use of the ultracentrifugation technique. Eleven of 12 subjects with the distinct α_2' peak, and 8 of 9 subjects with the unresolved α_2 area showed distinct quantitative abnormalities in the lipoprotein fractions, particularly in the –S 25-40 and –S 20-25 fractions, corresponding to α_2 and β globulins, respectively. The increases found in these lipoprotein fractions would indicate that at least a part of the atypical protein component represents lipoprotein. Whether lipoprotein accounts for the entire α_2' fraction or whether it represents one component of a heterogeneous mixture is unanswered.

The question whether the atypical component represents a split in the α_2 globulin fraction cannot be resolved on the basis of the data presented here. The α_2 globulin fraction represents an extremely heterogeneous mixture of components. An apparent split in this fraction could simply mean that an abnormal component or that components normally present in much smaller amounts are contributing qualitative differences in the α_2 electrophoretic picture.

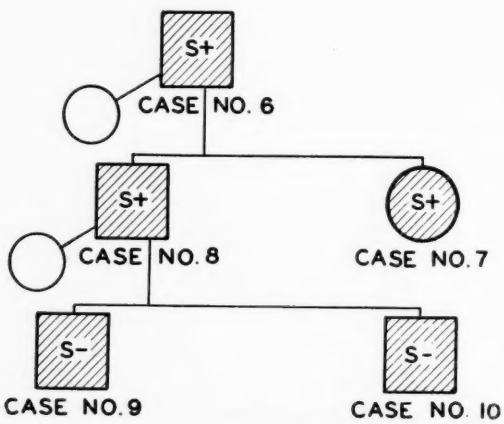


Fig. 2.—Correlation between inheritance pattern, clinical signs and symptoms of primary systemic amyloidosis, and atypical serum electrophoretic patterns. □ males, ○ females; ▨ ◍ atypical electrophoretic patterns; S+, positive clinical signs and symptoms; S–, negative clinical signs and symptoms.

There seems to be a definite correlation between the presence of clinical signs or symptoms of familial primary systemic amyloidosis and the atypical electrophoretic pattern. Of the subjects with normal electrophoretic patterns, none had clinical signs or symptoms of the disease. The great majority of those patients showing atypical electrophoretic patterns without clinical signs or symptoms were less than 18 years of age. The absence of any manifestation of primary systemic amyloidosis in this age group could be expected. On the basis of these results, it is felt that electrophoretic analysis might conceivably provide a means of predicting which of the younger members of this family with a history of primary systemic amyloidosis might be expected to develop manifestations of the disease.

This atypical electrophoretic pattern may also provide a means of following the inheritance pattern in this disease. The atypical patients listed in Table

I have been arranged according to immediate family groups (Patients 1 to 5, 6 to 10, 11 to 14, 15 to 16, 17 to 19, 20 to 21, 22 to 26, and 27 to 28). Patients 6 to 10 in Table I offer an example of the application of electrophoretic findings to the inheritance pattern of the disease. The correlation between atypical protein pattern and inheritance is outlined in Fig. 2.

Patient 6, a 52-year-old man, had an atypical electrophoretic pattern, with clinical signs and symptoms of the disease. His children, Patients 7 and 8, a 32- and a 22-year-old man and woman, also had atypical electrophoretic patterns and manifestations of the disease. The children of Patient 7, boys 9 and 6 years of age, also had atypical electrophoretic patterns but without either clinical signs or symptoms of the disease. It seems possible that these two subjects might subsequently develop signs of the disease.

Analysis of the inheritance pattern of the disease[4] in these 66 patients points to a simple dominant type of inheritance. This inheritance picture is clearly represented in the small group of cases shown in Fig. 2. It should be emphasized, however, that the determination of inheritance pattern was based on the results obtained on 66 subjects, covering three generations.

SUMMARY

Electrophoretic data on 66 members of a family with a history of primary systemic amyloidosis are discussed. Twenty-nine members of this group showed atypical electrophoretic findings. Fifteen of these 29 showed the presence of an atypical protein peak in the α_2 globulin region. Fourteen of the 29 were characterized by poor resolution in the α_2 globulin region.

There is evidence to indicate that at least a part of this atypical peak can be accounted for as lipoprotein.

A correlation exists between the incidence of clinical signs and symptoms of the disease and the existence of the atypical peak. All patients with manifestations of the disease had atypical electrophoretic patterns. Not all patients showing the atypical peak had manifestations of the disease, however.

It is hoped that a combination of electrophoretic findings with a knowledge of the inheritance pattern of the disease may provide another valuable diagnostic aid, as well as a possible means of predicting which members of the family might develop disease symptoms.

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ULTRACENTRIFUGAL ANALYSES OF SERUM LIPOPROTEINS IN FAMILIAL PRIMARY SYSTEMIC AMYLOIDOSIS

JOHN G. RUKAVINA, M.D., WALTER D. BLOCK, PH.D., AND ARTHUR C. CURTIS, M.D.
ANN ARBOR, MICH.

FAMILIAL systemic amyloidosis represents an only recently recognized facet of the primary type. Only four reports have appeared in the literature which emphasize the clinical, genetic, and pathologic features of this process.[1-4] No experimental biochemical data have been reported in the inherited expression of systemic amyloidosis.

Recently, Block, Rukavina, and Curtis[5] have published a preliminary report on the occurrence of an atypical serum electrophoretic peak, migrating between the α_2 and β globulin fractions, in five members of a large pedigree with the inherited form of the disease. This unusual finding has been further described in a subsequent report by these authors.[6]

The possibility that the unusual serum electrophoretic pattern might represent lipoprotein anomalies has been investigated. Serum lipoprotein analyses were carried out on 33 members of this family, with use of the ultracentrifugation technique. The diagnosis of this disease was based on clinical, laboratory, and pathologic data which will form the basis of a subsequent report.[7]

METHODS

Lipoproteins were analyzed by ultracentrifugation at a density of 1.21, according to the Lewis, Green, and Page[8] modification of Gofman's technique.[9] Cholesterol was determined by the method of Abell and associates.[10] Blood specimens were drawn at times when the influence of dietary factors on lipoprotein levels was minimal.

TABLE I. AVERAGE NORMAL HUMAN SERUM LIPOPROTEINS DETERMINED BY ULTRACENTRIFUGAL ANALYSIS AT DENSITY 1.21 (MODIFIED AFTER LEWIS, PAGE)

SEX	AGE	−S>70 (MG./100 ML.)	40-70 (MG./100 ML.)	25-40 (MG./100 ML.)	20-25 (MG./100 ML.)	1-10 (MG./100 ML.)
Male	18-34	36.7 ± 3.8*	24.2 ± 1.8		11.6 ± 1.1	153.7 ± 5.1
Male	35-60	59.5 ± 5.0	32.7 ± 1.8	<190	12.4 ± 0.8	155.8 ± 4.8
Female	18-34	21.8 ± 4.3	18.1 ± 1.4		11.6 ± 1.2	183.0 ± 8.4
Female	35-60	22.0 ± 5.0	29.1 ± 4.2	>180	13.3 ± 1.4	228.1 ± 11.6

*Standard error of the mean.

The average normal human serum lipoprotein values used as a standard of comparison were those of Lewis and Page[11, 12] (see Table I). These authors found that normal serum lipoprotein values vary both with age and with sex. These differences in normal values have been emphasized by other authors[13] and have been taken into account here wherever

From the Department of Dermatology and Syphilology, Medical School, and the Institute of Industrial Health, University of Michigan, Ann Arbor.
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figures are available. Unfortunately, no normal determinations were available in the literature for the 1- to 17-year age range. Several patients reported in the present paper fell into this age group. It has been assumed, therefore, that the normal values for this group are not greatly different from normal values for the 18- to 34-year age group. Those age groups for which normal lipoprotein values are available (18 to 34 and 35 to 60 years) indicate that these values increase with increasing age. On the basis of this fact, it might be speculated that normal values for the 1- to 17-year age group actually would be somewhat lower than those for the 18 to 34 group.

TABLE II. SERUM LIPOPROTEIN VALUES DETERMINED BY ULTRACENTRIFUGAL ANALYSIS AT DENSITY 1.21 IN 33 MEMBERS STUDIED FOR FAMILIAL PRIMARY SYSTEMATIC AMYLOIDOSIS

CASE NO.	SEX AND AGE	ATYPICAL SERUM PATTERNS	-S>70	40-70	25-40	20-25	1-10	SERUM CHOLESTEROL (MG./100 ML.)	REMARKS (SIGNS, SYMPTOMS, LABORATORY DATA, ETC.)
1	F, 58	Present	28	24	177	14	282	246	Neuropathy, skin biopsy
2	M, 32	Absent	<7	<7	141	19	199	215	Symptoms of neuropathy
3	F, 3	Absent	9	19	160	21	235	191	None
4	M, 20	Absent	<7	4	106	9	141	130	None
5	M, 7	Absent	6	24	152	7	141	129	None
6	F, 33	Present	7	7	152	14	177	179	Symptoms of neuropathy
7	M, 5	Present	*						None
8	M, 3	Present	†						None
9	F, 30	Present	7	9	129	14	152	147	Skin changes
10	M, 10	Absent	9	4	177	28	194	198	None
11	M, 52	Present	14	71	424	14	188	290	Neuropathy, eye changes, hepatomegaly
12	F, 22	Present	14	14	139	24	199	210	Neuropathy, splenomegaly
13	M, 32	Present	118	122	354	28	258	326	Anemia, hepatomegaly
14	M, 9	Present	19	14	235	<14	188	151	None
15	M, 6	Present	75	19	129	16	141	138	None
16	M, 52	Present	*						Neuropathy, eye changes, cardiomegaly
17	M, 22	Present	14	19	141	7	164	126	None
18	F, 17	Present	—	7	106	16	247	162	Negative
19	F, 24	Present	14	16	152	7	118	168	Negative
20	F, 47	Present	24	24	97	21	—	—	Neuropathy, heart failure, hoarseness
21	M, 21	Present	*						Negative
22	F, 64	Absent	14	28	398	<14	202	370	Neuropathy, hepatomegaly
23	F, 48	Present	24	55	290	12	133	266	Symptoms of neuropathy
24	F, 40	Absent	10	10	198	35	300	226	Neuropathy
25	F, 7	Absent	<7	9	157	12	188	159	None
26	M, 12	Present	7	9	223	14	129	168	None
27	F, 18	Present	108	24	164	14	118	158	None
28	F, 73	Present	*						Symptoms of neuropathy
29	M, 46	Present	12	35	280	19	235	226	Symptoms of neuropathy
30	F, 41	Absent	12	7	177	14	171	203	None
31	M, 32	Absent	7	16	188	9	106	178	None
32	M, 46	Present	9	52	280	12	100	233	Neuropathy, skin changes, hepatomegaly
33	F, 17	Present	<7	7	152	19	141	181	None
34	M, 16	Present	4	7	118	7	118	165	None
35	F, 13	Present	26	14	152	7	101	184	None
36	F, 12	Present	*						None
37	M, 44	Absent	56	80	424	<14	141	240	Hepatomegaly, gastrointestinal
38	M, 69	Present	64	35	218	16	141	221	Neuropathy, cardiac insufficiency
39	M, 39	Present	*						Neuropathy
40	M, 73	Present	4	16	141	7	106	242	Neuropathy, cardiac insufficiency

*Lipoprotein determination not done.
†Material insufficient for analysis.

RESULTS

Liproprotein determinations were carried out on 33 members of this pedigree.[1] The lipoproteins of all members studied showed all of the components present in normal sera (–S>70, 40-70, 25-40, 20-25, and 1-10) (Table II). Lipoprotein concentrations were found to be within normal limits in 6 cases. In 3 of these cases (4, 5, and 31), all other data obtained were also normal and the subjects were considered free of systemic amyloidosis. In the 3 remaining cases (19, 34, 40) with normal lipoprotein values, electrophoretic abnormalities were demonstrated.

Twenty-seven of the 33 subjects demonstrated abnormal lipoprotein values. Of this number, 15 were females, 12 were males. Ages ranged from 3 to 73 years; the females ranging from 3 to 73 years, and males from 6 to 73 years. The mean age for the females was 33 years and for the males 32 years. Fifteen of the 27 cases were below 33 years of age.

Analysis of the data showed abnormalities in all components of the sera studied. The –S>70 fraction had the least number of elevations (Cases 13, 15, 27). The –S 40-70 component was elevated in four males (Cases 11, 13, 32, 37). Only two females showed significant elevations in this fraction.

A rather consistent pattern of abnormality existed in the –S 25-40, 20-25, and 1-10 fractions. In the –S 25-40 concentration, abnormal values were found in 8 males (Cases 11, 13, 14, 26, 29, 32, 37, 38) and in 7 females (Cases 3, 22, 23, 25, 27, 33, 35). These elevations were very marked in two males, aged 6 and 12 years.

The –S 20-25 level was elevated in 9 males (Cases 2, 10, 11, 13, 14, 15, 26, 29, 38) and in 10 females (Cases 3, 6, 9, 12, 18, 20, 24, 27, 30, 33). Three of the affected males were 6, 10, and 12 years of age, respectively. Seven of the 10 females were under 35 years of age, the youngest being a 3-year-old child.

Appreciable increases in the measurable concentrations of the –S 1-10 fraction were noted in 7 males (Cases 2, 10, 11, 13, 14, 17, 29) and in 5 females (Cases 1, 3, 12, 18, 24). The youngest male in this group was 9 years of age and the youngest female 3 years of age. The serum cholesterol levels were elevated in only 5 cases (1, 11, 13, 22, 23).

DISCUSSION

The genetic profile of this pedigree indicates a dominant form of inheritance for the disease.[14] Interesting correlations were found between lipoprotein abnormalities and the inheritance pattern. The data indicate varying degrees of genetic lipoprotein impactness.

The offspring of Patient 11, consisting of a daughter (Patient 12), son (Patient 13) and two grandsons (Patients 14, 15) showed the most marked abnormalities in lipoprotein values. One member of this group (Patient 13) showed remarkable elevation in all of the lipoprotein fractions. His sons, aged 6 and 9 years, respectively, likewise demonstrated unusual changes in the –S components.

The offspring of Patient 1, embracing a son (Patient 2), two daughters (Patients 6, 9), two grandsons (Patients 4, 5), and a granddaughter (Patient 3), revealed appreciable concentrations in the –S 20-25 and 1-10 fractions. Three members of this group (Patients 3, 4, 5) were entirely free of the disease by all methods of investigation. The most significantly affected member of this unit was a 3-year-old child.

The offspring of Patient 32, made up of three daughters (Patients 33, 35, 36) and a son (Patient 34) exhibited changes preponderantly in the –S 25-40 fraction. A brother (Patient 37) showed similar changes.

There was no significant pattern of serum cholesterol elevation in any of the family segments studied.

The clinical data were next related to the results secured by ultracentrifugation. In 16 cases an abnormal concentration of one or more –S fractions was accompanied by a clinical expression of the disease. There was no sex preponderance noted in this correlation. Significantly, 11 apparently normal members, as determined by clinical study, demonstrated a definite increase in this class of molecules. Of this latter group, 9 were under 18 years of age.

The data secured by ultracentrifugation were correlated with those obtained by free electrophoresis. In 12 Patients wherein demonstrable quantitative evidence of an α_2' peak was found, 10 Patients (6, 11, 12, 13, 14, 15, 17, 18, 20, 23) showed abnormal lipoprotein levels in the –S 25-40 and 20-25 fractions. In those instances wherein the α_2 electrophoretic peak was poorly resolved, an increase in the measurable concentrations of the –S fractions was found in 9 Patients (1, 9, 26, 27, 29, 32, 33, 35, 38). In 8 Patients with normal electrophoretic patterns, lipoprotein components were found to be elevated.

The lipoprotein patterns of this family group appear to be unusual. Lewis and Page[12] found the –S 20-25 components to be normal, while lower than normal values were obtained in the –S 25-40 and 1-10 fractions in their cases of multiple myeloma. These same workers in another report,[11] demonstrated a marked increase in the –S>70, 40-70, 25-40, and 20-25 lipoprotein fractions in the nephrotic phase of glomerulonephritis. In none of the cases of the present study was there any clinical or laboratory evidence of multiple myeloma, renal insufficiency, or malignant hypertension. In only 3 patients was there suggestive laboratory evidence of hepatic derangement.

SUMMARY

The serum lipoprotein fractions of 33 members of a large family pedigree affected by an inherited form of systemic amyloidosis have been analyzed by ultracentrifugation.

In 27 patients elevations of the –S fractions were found. Consistent elevations were found particularly in the –S 25-40 (β_1-lipoprotein), 20-25 (α_2-lipoprotein), and 1-10 (α_1-lipoprotein) components.

The lipoprotein genetic pattern suggests that varying degrees of inheritance impactness are manifest.

There appears to be a correlation between the clinical expression of the disease and lipoprotein changes. That apparently normal subjects may demonstrate lipoprotein abnormalities has been emphasized.

A quantitative and qualitative correlation between atypical electrophoretic pattern and increased flotation fractions was noted in 19 of 27 patients studied.

The data suggest that in this pedigree systemic amyloidosis represents, at least in part, an anomaly of lipoprotein metabolism.

We are indebted to Drs. Irvine H. Page and Lena Lewis for carrying out the ultracentrifugal lipoprotein analyses in their laboratory. We wish to thank Dr. C. E. Jackson, Caylor-Nickel Clinic, Bluffton, Indiana, for securing the blood specimen for this study.

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STUDIES ON GROWTH HORMONE

I. The Effects of Growth Hormone and Insulin Upon the Synthesis of Protein in Perfused Livers

John F. Prudden, M.D., Med. Sc. D.,* M. Kendall Young, Jr., M.A., and Jerry A. Stirman, M.D.**
Fort Sam Houston, Tex.

THE mechanisms by which growth hormone† and insulin exert their effects on protein, fat, and carbohydrate metabolism are not yet well defined, nor has their clinical effectiveness been assayed in circumstances of malnutrition and/or posttraumatic cachexia. The current series of papers represent the beginning of such a systematic study. This first publication reports on liver perfusion studies done with the major aim of quantitating the effect of the hormones on protein metabolism, with additional consideration of their effects on fat and carbohydrate metabolism. The second and third papers in this series report clinical studies of the effect of growth hormone‡ on the metabolic balance and on the incorporation of S^{35}-labeled methionine into the plasma proteins of convalescent burn patients, respectively.

The study reported here was undertaken to provide a quantitative estimate of the degree to which protein metabolism could be shifted in the direction of anabolism by the addition of growth hormone and/or insulin to the perfusate being employed.

METHODS

A. *The Apparatus.*—Our perfusion apparatus is described completely elsewhere.[1]

B. *The Hepatectomy.*—The entire procedure from the procurement of blood from donor rabbits through the hepatectomy and the assembling of the apparatus is carried out with the greatest possible effort to avoid bacterial contamination. The receptacle for donor blood is autoclaved, as is the tubing through which the blood is drawn. The hepatectomy is carried out precisely as though the procedure were being done clinically, with the exception that the operators are not gowned. (All instruments, drapes, sutures, etc., are autoclaved or chemically sterilized, and the rabbit's abdomen is shaved and prepared prior to draping.) The various pieces of the perfusion apparatus are packaged and autoclaved individually and then reassembled as a unit by an assistant wearing sterile gloves. The tubing is thoroughly cleansed of old perfusate after each run and soaked in alcohol prior to autoclaving.

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*Formerly Captain, MC, USA. Present address: Department of Surgery, Columbia-Presbyterian Medical Center, New York, N.Y.

**Formerly Captain, MC, USA. Present address: Department of Surgery, Southwestern Medical School, Dallas, Tex.

†We are indebted to Dr. Irby M. Bunding of the Armour Laboratories for the growth hormone used in this study. This growth hormone lot (Armour #R491024) was reported to contain 0.32 ± 0.098 units of thyrotropin per milligram with a potency of 64 ± 33 per cent of the Armour Standard 22 KRZ preparation, as measured by body weight increase in hypophysectomized female rats.

‡For brevity, we shall refer to growth hormone as GH throughout this paper.

C. *The Perfusate.*—The collection of the donor blood and the preparation of the amino acid solution is done according to the method previously described.[1] The solution was made up of a 50-50 mixture of defibrinated rabbit blood and an amino acid mixture* containing the essential acids and a few others, as indicated in Table I. The calculated millimolarity appears somewhat high. However, no evidence of cellular dehydration was observed in the microscopic sections; the tendency being toward a slight edema.[1] When perfusate is placed across a semipermeable Visking membrane from rabbit plasma, there is no detectable movement of fluid.

TABLE I. APPROXIMATE MILLIMOLARITY OF THE PERFUSATE

	MILLIMOLES/LITER
A. Amino acids:	
1. Arginine	2.55
2. Glycine	54.40
3. l-Histidine	4.81
4. Iso-leucine	10.83
5. Leucine	22.34
6. Lysine	11.42
7. l-Methionine	4.80
8. Phenylalanine	5.70
9. l-Threonine	2.84
10. dl-Tryptophane	1.57
11. l-Valine	10.62
12. l-Glutamic acid	1.82
B. Other constituents:	
13. Lactic acid	19.70
*14. Na^+	133.00
*15. Cl^-	107.00
*16. K^+	4.50
*17. CO_2	1.80
Total millimolarity	399.70

*By analysis, remainder by calculation.

D. *The Experimental Protocol.*—Table II illustrates the unchanging portion of the experimental procedure. When different experiments are being run, minor additions are made. For example, for the evaluation of growth hormone, 50 mg. of GH in 5 ml. of saline is added to the perfusate at the start of the perfusion. When an insulin experiment is done, 40 units of regular insulin are added at the start of the experiment and again at six hours. When GH and insulin are being tested together, the two protocols are combined. At the end of the perfusion, the liver is removed from the organ chamber and carefully weighed. Next, three blocks of tissue are removed from various lobes and fixed in 10 per cent Formalin prior to sectioning and staining. Hematoxylin and eosin and Schiff's glycogen stains are done on this material. The remainder of the liver is placed in a suitably labeled beaker and stored in the Deepfreeze until ready for analysis, as indicated below. The reservoir is also removed from the apparatus at the end of the run,† sealed, and stored at 4 degrees centigrade. Each hourly perfusate (12 ml.) sample is placed in a 15 ml. centrifuge tube containing a small amount of NaF (except when Na^+ is to be measured), to prevent enzymatic activity. The tubes are stored overnight at 4 degrees centigrade prior to analysis.

*The definitely higher levels obtained with the biuret reaction than by the micro-Kjeldahl technique (see results below), as well as the differential movement of these analyses with time, leads one to conclude that peptide formation occurs under these experimental conditions (see below). The amino acid solution prior to mixing gave no biuret reaction, a fact which appears to be in accord with this point of view. Since peptide formation occurs in the perfusate, it is clear that the calculated millimolarity will be an inaccurate reflection of the osmotic pressure of the perfusate.

†In the interests of brevity, the experiments described in this paper will be referred to as "runs." Thus, control experiments or ones with growth hormone or insulin are called control, GH, or insulin runs.

TABLE II. THE EXPERIMENTAL PROTOCOL

TIME IN HOURS	
0	Hematocrit, culture, perfusate sample, pH, 4 ml. heparin solution*
1	Perfusate sample, pH
2	Perfusate sample, pH
3	Perfusion sample, pH, 4 ml. heparin solution*
4	Perfusate sample, pH, change CO_2 traps, culture, Aureomycin, 25 mg.
5	Perfusate sample, pH
6	Hematocrit, perfusate sample, pH, 4 ml. heparin solution*
7	Perfusate sample, pH
8	Perfusate sample, pH, change CO_2 traps, culture, Aureomycin, 25 mg.
9	Perfusate sample, pH, 4 ml. heparin solution*
10	Perfusate sample, pH
11	Perfusate sample, pH
12	Hematocrit, perfusate sample, pH, collect CO_2 traps, remove liver and weigh, obtain tissue blocks from three hepatic lobes and place in 10 per cent Formalin, remove perfusion reservoir, seal, and store at 4° C. Remainder of liver placed in Deepfreeze.

*Contains 1,000 units per milliliter (~10 mg./ml.)

E. *Chemical Procedures.*—Following separation of the red cells, the perfusate "plasma" was analyzed by the following techniques.

1. Urea: Gentskow and Masen[2]
2. Glucose: Folin and Wu[3]
3. Perfusate protein:
   a. Biuret method; Gornall and associates[4]
   b. Micro-Kjeldahl technique; Ma and Zuazaga[5]

Suitable corrections were made in both of these protein techniques for the hemoglobin concentration in the perfusate "plasma." The technique involved measurement of the pigment with the standard acid hematin technique and quantitation of the protein equivalent of hemoglobin in the case of the biuret reaction.[6] It was demonstrated[6] that hemoglobin goes with the albumin fraction in the sodium sulfate fractionation employed in the biuret reaction.

4. Alpha amino nitrogen: Albanese[7] and Fister.[8]

5. Carbon dioxide: The CO_2 respired by the liver is titrated with 0.1 N HCl in the NaOH employed in the CO_2 traps. The volume of the trap was then used to calculate the total amount given off.

6. Hepatic protein: Carefully weighed samples of liver were homogenized at appropriate dilution, the protein precipitated with trichloracetic acid, separated, dried, and reweighed. The total liver protein and per cent protein were then calculated with the employment of this result and the liver weight.

7. Total lipid: About 200 mg. of liver were weighed into a container and dried to constant weight at 70° C. The fat was then extracted with a 3:1 mixture of absolute alcohol and ether in a capped container for 48 hours at 37° C. Next the supernatant was discarded and the sample dried to constant weight at 70° C. The total hepatic lipid and per cent lipid were calculated from these results.

8. Glycogen: The hepatic glycogen was determined by the employment of a modified anthrone reagent,[9] in the method of Sefter and associates.[10]

F. *Isotopic Procedures.*—

1. Plasma proteins:* The proteins were precipitated in duplicate with trichloracetic acid in a 1:10 dilution, resuspended, and washed a total of five times to rid them of supernatant radioactivity. They were then resuspended in acetone and filtered onto preweighed No. 50 Whatman paper. After careful drying at 70° C., the filter papers were weighed and their activity estimated in a methane flow counter with a decade type scaler. The zero time

*For simplicity, the perfusate proteins are termed plasma proteins throughout this paper.

sample served as background. Suitable corrections were made for absorption with the employment of an absorption curve worked out in this laboratory. The absorption curve of protein is contrasted with that of $BaC^{14}O_3$* in Fig. 1.

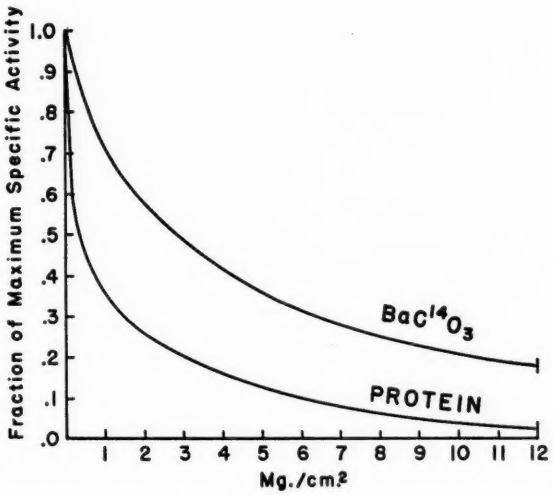

Fig. 1.—The absorption curves for C^{14} in plasma and hepatic protein and for $BaC^{14}O_3$.

2. Liver protein: Essentially the same technique was employed with the liver proteins, except that larger aliquots of a liver homogenate were employed in the interests of greater precision, since only one sample was available rather than hourly ones, as in the case of the plasma proteins.

3. Estimation of the isotopic dose: Five milligrams of glycine-2-C^{14} of a specific activity of 1.45 mc. per millimole was weighed out, 5 ml. distilled water added, then 4 ml. added to the perfusate, and 0.5 ml. diluted to 250 ml. for use as the water standard. The radioactivity so measured enabled a calculation of the total injected counts per minute to be made. Appropriate correction was made for the absorption of radioactivity in the trichloracetic acid solution remaining after precipitation of the proteins in the zero time sample, and this correction factor (F_1) was then appropriately applied to the counting rates. It has been demonstrated that the same correction factor applies whether or not the red cells have been lyzed by alternate freezing and thawing. It is therefore possible to count a lyzed 12-hour sample, calculate the total counts per minute remaining in the plasma supernate at that time, add to it the total counts in plasma protein, in liver protein, in liver supernate (see below), and in the CO_2 traps (see below), and then to compare it with the counts per minute injected. In this manner, the recovery of injected counts may be ascertained (see Table III).

4. Perfusate and liver supernates: The perfusate supernates were plated in appropriate dilution on copper in 200 lambda quantities and counted as indicated above; and the liver supernates were handled in exactly the same manner. After correction for dilution, the corrected rates were multiplied by the absorption factor (F_1) (see above), which gave the total counts per minute in these compartments.

5. **CO_2 traps:** The $C^{14}O_2$ was precipitated as $BaC^{14}O_3$, filtered onto paper, dried, resuspended in methyl alcohol, and plated on copper by drying in an apparatus similar to that described by Calvin and associates.[11] The counting rate was then corrected for absorption (see Fig. 1) and for the fraction of CO_2 in the trap which was actually counted to give the total counts per minute in the CO_2 trap. The totals for each trap were then added to give the total in all traps.

*These results with $BaC^{14}O_3$ almost exactly reproduce those of Calvin and associates.[11]

TABLE III. SUMMARY—PERFUSION RESULTS AND TESTS

COMPARISON	A. PERFUSATE ALBUMIN (BIURET) LARGER	P VALUES*		B. PERFUSATE TOTAL PROTEINS (BIURET) LARGER	P VALUES*	
Controls vs. GH	GH	<0.05	>0.025	Control	<0.05	>0.025
Controls vs. insulin	Insulin	<0.6	>0.5	Insulin	<0.1	>0.05
Controls vs. insulin + GH	Controls	<0.025	>0.02	Insulin + GH		<0.001
GH vs. insulin + GH	GH		<0.001	Insulin + GH		<0.001
GH vs. insulin	GH	<0.4	>0.3	Insulin	<0.02	>0.01
Insulin vs. insulin + GH	Insulin	<0.1	>0.05	Insulin + GH		<0.001

COMPARISON	C. PERFUSATE TOTAL PROTEINS (MICRO-KJELDAHL) LARGER	P VALUES*		D. PERFUSATE UREA LARGER	P VALUES*	
Controls vs. GH	Control	<0.005	>0.001	Controls	<0.25	>0.2
Controls vs. insulin	Control		<0.001	Insulin	<0.01	>0.005
Controls vs. insulin + GH	Control		<0.001	Insulin + GH		<0.001
GH vs. insulin + GH	GH		<0.001	Insulin + GH		<0.001
GH vs. insulin	GH	<0.6	>0.5	Insulin		<0.001
Insulin vs. insulin + GH	Insulin		<0.001	Insulin + GH	<0.9	>0.8

COMPARISON	E. PERFUSATE PROTEIN ACTIVITY LARGER	P VALUES*		F. PERFUSATE ALPHA AMINO NITROGEN LARGER	P VALUES*	
Controls vs. GH	GH		<0.001	Controls	<0.01	>0.005
Controls vs. insulin	Insulin		<0.001	Insulin	<0.05	>0.025
Controls vs. insulin + GH	Insulin + GH		<0.001	Insulin + GH	<0.4	>0.3
GH vs. insulin + GH	Insulin + GH	<0.01	>0.005	Insulin + GH		<0.001
GH vs. insulin	Insulin	<0.005	>0.001	Insulin		<0.001
Insulin vs. insulin + GH	Insulin	<0.2	>0.1	Insulin	<0.1	>0.05

*It was possible to pair the hourly means of the various groups in these particular categories; hence, these p values are calculated on the basis of a paired comparison using the "t" test.

COMPARISON	G. HEPATIC PROTEIN CONTENT (AS % WET WEIGHT) MEANS	P VALUE		H. HEPATIC TOTAL LIPID CONTENT MEANS	P VALUE		I. HEPATIC GLYCOGEN CONTENT MEANS	P VALUE	
Controls vs. GH	12.97 vs. 15.56	<0.001		3.815 vs. 3.710	<0.9	>0.8	0.195 vs. 0.381	<0.2	>0.1
GH + insulin vs. control	14.73 vs. 12.97	<0.4	>0.3	5.320 vs. 3.815	<0.4	>0.3	0.274 vs. 0.195	<0.1	>0.05
Insulin vs. control	12.87 vs. 12.97		>0.9	4.620 vs. 3.815	<0.5	>0.4	0.324 vs. 0.195	<0.2	>0.1
GH vs. insulin	15.56 vs. 12.87	<0.05	>0.025	3.710 vs. 4.620	<0.5	>0.4	0.380 vs. 0.320	<0.9	>0.8
GH vs. insulin + GH	15.56 vs. 14.73	<0.8	>0.7	3.710 vs. 5.320	<0.4	>0.3	0.380 vs. 0.270	<0.4	>0.3
Insulin vs. insulin + GH	12.87 vs. 14.73	<0.4	>0.3	4.620 vs. 5.320	<0.6	>0.7	0.320 vs. 0.270	<0.5	>0.4
All insulin runs vs. all noninsulin runs				5.010 vs. 3.770	<0.25	>0.2			

TABLE III.—CONT'D

COMPARISON	J. CO_2 EXPIRED		K. LIVER SUPERNATES*		L. HEPATIC PROTEIN ACTIVITY	
	MEANS IN ML./HR.	P VALUE	MEANS AS % COUNTS	P VALUE	MEANS IN % COUNTS	P VALUE BY "T" TEST
Controls vs. GH	74.40 vs. 59.67	$<0.5\ >0.4$	7.50 vs. 6.18	$<0.9\ >0.8$	0.49 vs. 1.48	<0.001
Controls vs. insulin + GH	74.40 vs. 89.59	$<0.6\ >0.5$	7.50 vs. 6.99	>0.9	0.49 vs. 1.22	$<0.02\ >0.01$
Controls vs. insulin	74.40 vs. 65.53	$<0.9\ >0.8$	7.50 vs. 10.52	$<0.2\ >0.1$	0.49 vs. 1.25	$<0.05\ >0.025$
GH vs. insulin + GH	59.67 vs. 89.59	$<0.2\ >0.1$	6.18 vs. 6.99	>0.9	1.48 vs. 1.22	$<0.25\ >0.2$
Insulin vs. GH	65.53 vs. 59.67	$<0.6\ >0.5$	10.52 vs. 6.18	$<0.05\ >0.025$	1.25 vs. 1.48	$<0.5\ >0.4$
Insulin vs. insulin + GH	65.53 vs. 89.59	$<0.5\ >0.4$	10.52 vs. 6.99	$<0.1\ >0.05$	1.25 vs. 1.22	>0.9
Insulin alone vs. all other determinations			10.52 vs. 6.95	$<0.02\ >0.01$		

*The activity remaining in the supernate after precipitation of the hepatic protein with 10 per cent trichloracetic acid.

M. PER CENT OF COUNTS IN CO_2 TRAPS

COMPARISON	MEANS	P VALUE
Controls vs. GH	0.30 vs. 0.60	$<0.4\ >0.3$
Controls vs. insulin + GH	0.30 vs. 1.20	$<0.05\ >0.025$
Controls vs. insulin	0.30 vs. 0.58	$<0.2\ >0.1$
GH vs. insulin	0.60 vs. 0.58	>0.9
GH vs. insulin + GH	0.60 vs. 1.20	$<0.2\ >0.1$
Insulin vs. insulin + GH	0.58 vs. 1.20	$<0.1\ >0.05$

N. PER CENT RECOVERY OF INJECTED COUNTS—98.61%

$\hat{\sigma} = 5.13\%$
$\hat{\sigma} = 1.42\%$

O. NORMAL HEPATIC CONTENT

CONSTITUENT	MEANS AS % NET WEIGHT	STANDARD DEVIATION
Protein	14.06	2.14
Total Lipid	5.61	1.50
Glycogen	0.30	0.32

RESULTS

The results obtained in the total of 23 runs, consisting of 11 controls, 6 GH runs, 3 insulin perfusions, and 3 runs in which GH and insulin were both added, are presented in Table III. The nonradioactive chemical results are

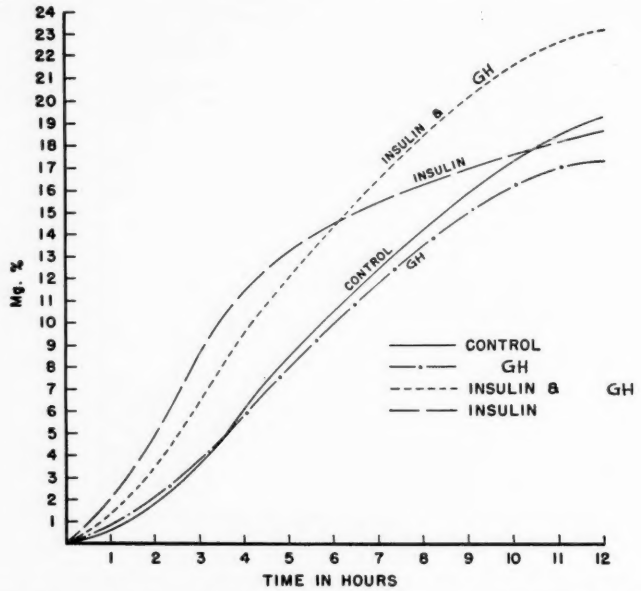

Fig. 2.—The composite urea curves exhibiting the tendency for insulin to increase the production of urea and for GH to diminish it.

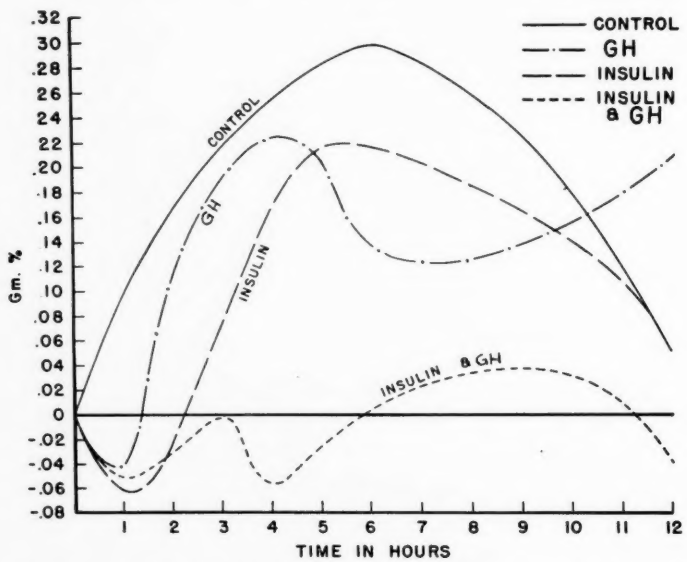

Fig. 3.—The course of the total protein concentrations in the plasma over time in the various experimental groups. Notice the rising tendency exhibited for the GH group from the seventh hour on while the other curves were falling.

calculated on the basis of all of the perfusions. The C^{14} techniques were applied to 4 controls, 3 growth hormone runs, 3 insulin perfusions, and 3

growth-hormone-plus-insulin runs. The probability for identity between the types of perfusion being compared are given under the column headed "p" values. In those instances (categories A to F) where hourly samples were available for comparison, the means of the various samples for each hour in each

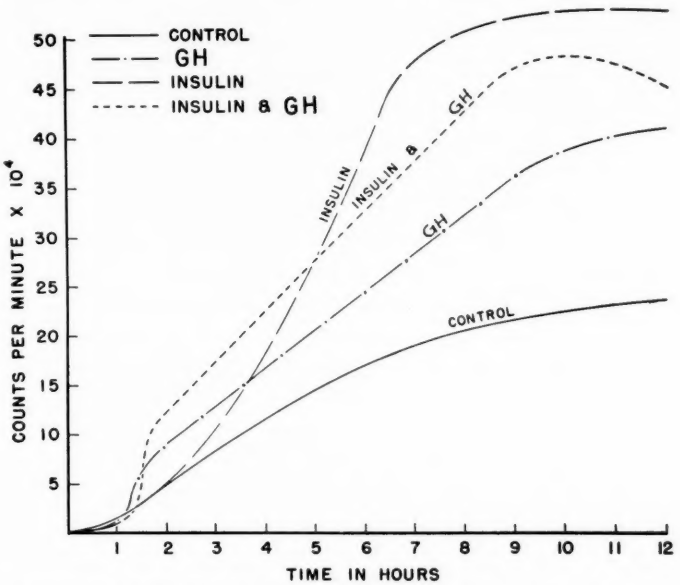

Fig. 4.—The composite total perfusate protein activities indicate the definitely higher activities occurring in the presence of insulin. GH also causes a definite increase over the controls.

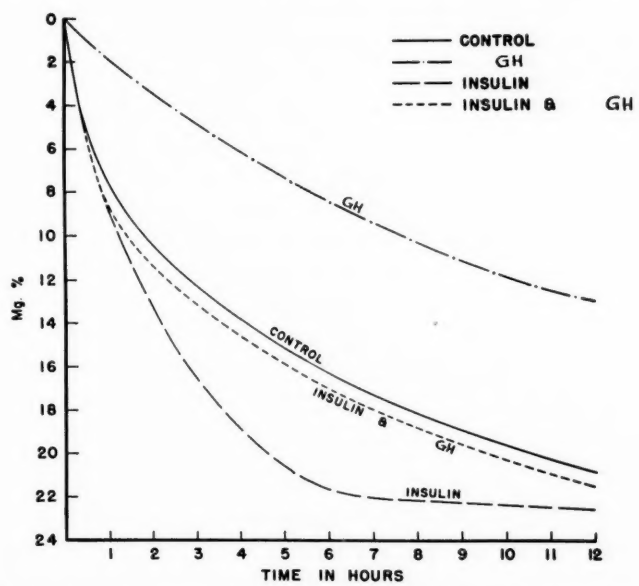

Fig. 5.—The alpha amino nitrogen curves show a markedly lessened decrease in this chemical fraction in the presence of GH. Insulin definitely causes larger and more rapid falls.

category were compared by means of a "t" test, with pairing of the twelve hourly means against one another. In all comparisons, the values were "normalized" by

converting the hourly samples to increments or decrements from a starting level of zero. The movement of the constituents compared in categories A to F are shown in Figs. 2 through 5. The starting concentrations of most constituents were quite consistent. The means and standard deviations were as follows:

1. Albumin	1.735	±	0.198	Gm. per cent
2. Total protein (biuret)	2.667	±	0.320	Gm. per cent
3. Total protein (micro-Kjeldahl)	2.231	±	0.285	Gm. per cent
4. Urea	6.537	±	1.98	mg. per cent
5. Alpha amino nitrogen	171.4	±	15.44	mg. per cent

Perfusate protein activity was initially zero.

In categories G to M of Table III, the comparisons were between characteristics of the various types of perfusions at the end of the twelve-hour period of perfusion. Here only one result was available for each perfusion; hence, the values for the categories under consideration were compared by means of a "t" test without pairing.

The last two categories (N and O) show the per cent recovery of injected radioactivity and the constituent values for normal rabbit liver.

DISCUSSION

These data indicate that growth hormone is a rather powerful anabolic agent. It was the most efficient agent tested in effecting the synthesis of tissue (hepatic) protein. The hormone caused a slight over-all increment in chemical "plasma" protein (with an impressive continuous rise, beginning at eight hours), and the uptake of glycine-2-C^{14} into this protein was increased. The comparative magnitude of the "plasma" protein and the tissue protein results (in the GH runs) appear to show that plasma protein had been drawn upon in order that more tissue protein might be synthesized. This apparently indicates that the liver utilized plasma protein in order to make tissue protein, as well as amino acids, for a direct cellular synthesis. This corresponds with the presumed chain of events in the periphery. In addition, the uptake figures for plasma protein in the insulin runs are significantly higher than those for the growth hormone group without clear-cut differences in the chemical levels. This, in conjunction with the increased production of urea in all insulin runs, would point to a higher turnover rate* for plasma protein under the influence of insulin. Furthermore, in view of the highly significant increase in plasma protein C^{14} incorporation in the GH runs in comparison with the controls, coupled with an apparently decreased production of urea in the former group, one may also conclude that the GH group's protein turnover rate was slower than in all other types of runs, including the controls. The decreased production of urea in these experiments

*_Turnover_ is used here in a less precise way than in circumstances where a steady state exists. The isotopic data permits comparisons of the rates of uptake and loss from protein of amino acids in the various experimental groups. They do not always indicate whether this is due to actual protein synthesis and breakdown or only to the opening of peptide linkages and exchange of amino acids within essentially complete proteins. The chemical data, however, provide us with rather firm proof that the traffic in and out of the plasma protein pool (turnovers in the sense indicated above) is largely referable to synthesis and breakdown. It should be understood that the uptake of an amino acid does not count toward turnover, for it (or its brother) must be lost to the protein to have completed a turnover in this sense. A protein molecule will not have gone through a complete turnover until its starting amino acid components have been completely replaced by newcomers; therefore, we are generalizing concerning the *mean molecular turnover rate of protein amino acids.*

is in accordance with previous observations[12] and with the recent demonstration of the inhibition by GH of arginase and other urea-producing hepaitc enzymes[13] (see Fig. 2).

The albumin level (by biuret) was best maintained by GH, despite the fact that the total proteins (biuret) were lowest in that group. At first glance, this seems a surprising discrepancy; but it will be seen below that this is probably due to the movement of the polypeptides with the globulin fraction in the sodium sulfate fractionation of plasma protein in the biuret procedure. The GH runs supplemented with insulin exhibited the highest total proteins by the latter technique, with insulin as the intermediate. This cannot be interpreted without considering the rather striking changes in these relationships which occurred when the same samples were analyzed by the micro-Kjeldahl method. Here the total protein in the control group was significantly higher than in the other experiments, and the GH perfusions were next highest. The insulin group was essentially the same as the GH runs; but the insulin and GH runs, which showed definite increases over all others when the protein levels were estimated with the biuret reaction, exhibited the lowest protein level by micro-Kjeldahl. Furthermore, as discussed in more detail below, the movement of these constituents with time must be considered (as illustrated in Fig. 3). It is seen there that the GH group had distinctly the highest level at twelve hours. This level was significantly elevated over those of the other groups. The consistency of the various samples which went into each hourly mean were responsible for the statistical significance of the differences.

What is the significance of this reverse in order of the total protein concentrations when one analyzes the same samples by biuret and then by micro-Kjeldahl? It is considered that this circumstance is directly referable to the fact that the biuret reaction measures polypeptides as well as protein (or, more accurately, more so than does the Kjeldahl). If this is the reason, then one will infer from these data that there is a considerably greater concentration of polypeptides present in the perfusate of the insulin group than in those of the GH or control group. Furthermore, one must bear in mind that polypeptides may be present because they are being built up continually toward protein, because they are remnants of catabolized protein molecules, or because they represent polypeptides which cannot go on to protein under the existing experimental conditions. If the first possibility were the case, one might anticipate that the groups exhibiting the highest biuret values would also show the highest protein levels by the Kjeldahl method. This is not the case; therefore, we must assume that one of the two latter circumstances exist.

The conclusion from this evidence would appear to be that GH will maintain the plasma protein level at a higher value than will insulin or insulin in combination with GH, at the same time that it is stimulating the production of more hepatic protein. It is true that the control group plasma proteins were significantly higher than were those of the GH runs by micro-Kjeldahl; however, the hepatic proteins were considerably lower (see above). Furthermore, it must be remembered that the comparison in Table III is based on a "t"

test, with pairing between the hourly sample means in the two groups. This statistical treatment can obscure differences if the trends are not essentially the same with respect to time or magnify them if the opposite is true.* Throughout this study, this problem will be noted if it exists. In the case of the control and GH plasma protein levels as estimated by micro-Kjeldahl, this situation did exist. The GH group showed a consistently lower total protein throughout most of the twelve-hour period of perfusion, but with a narrowing gap, until at eleven and twelve hours the circumstances were reversed (see Fig. 3). At twelve hours the mean total protein of the GH group was 0.210 Gm. per cent greater than at the start, and that of the control group had receded to only 0.050 Gm. per cent above the starting level after a peak value of +0.228 Gm. per cent at three hours. Interestingly enough, the final GH level was the peak value in that group. The difference between these two twelve-hour means is highly significant ($0.01 > p > 0.005$). The insulin groups followed the same general trend as the controls (at a lower level), ending at +0.047 Gm. per cent (insulin) and 0.040 Gm. per cent (insulin and GH), respectively.

Therefore, we see that at the conclusion of the experiment, the GH group had a higher plasma protein level, a higher hepatic protein content, and a lower urea production than the other experiments. The plasma protein uptake was higher in the insulin group, with an apparent GH inhibition of this particular insulin effect being manifest.† Coupled with the chemical findings, these indicate that the turnover of the plasma protein in the insulin runs was most rapid, that GH had an inhibiting effect on this rate, and indeed that the GH group rates were slowest of all. These data thus appear to indicate that not only is the hormone anabolic in the positive sense but it appears also to inhibit catabolism. The latter statement is based on the assumption that the amounts of urea produced in a closed but viable system is a valid measure of the protein catabolic rate.

Quite another interpretive problem is raised when one compares the falls in alpha amino nitrogen from group to group (see Fig. 5). It is evident that the insulin group exhibited a considerably greater fall than did the GH and control runs. Furthermore, GH administered concomitantly with insulin exerted an inhibiting effect on this fall. In addition, the fall in the insulin group's alpha amino nitrogen was greater than the falls exhibited in the GH and control runs by a margin larger than could be accounted for in the urea nitrogen increase which the insulin group of experiments exhibited (10.0 mg. per cent of alpha amino nitrogen versus about 4 mg. per cent of urea nitrogen at 12 hours). This appears to indicate that the amino acid nitrogen so utilized is in the polypeptide form, since it cannot be accounted for in its original form,

*This statistical analysis evaluates the *relative* trends in protein levels of the several groups of experiments. A "t" test of absolute levels, in contradistinction to one of concentration differences, would introduce an element of spurious variability into the data due to the graphical trends with time. These time variations are susceptible of more complete analyses as to time-dependence equations, rate-constants, etc. These studies will appear in a subsequent publication.

†An interesting observation may be made regarding the character of the uptake curves. When a curve peaks and the level then falls off as does the insulin and GH group, one can conclude immediately that the protein is turning over more rapidly than groups which have not yet peaked. This conclusion is borne out by the fact that this group has the lowest chemical protein levels and the highest urea production. Thus, the fact that this is a closed system permits the use of the chemical values in the same manner as isotopes in estimating turnover (see Fig. 4).

in protein (by Kjeldahl), or in urea. This is directionally confirmed by the increases in the insulin runs' total protein values by biuret, as indicated above.

It is therefore considered that the higher disappearance rate of alpha amino nitrogen in the insulin group is due to peptidization of the amino acids short of complete protein, as well as through deamination with conversion to urea nitrogen. The very great increase in glucose (see Table IV) in all insulin runs would appear to be in accord with the latter process. It is presumed that the glucose levels are referable in part to deamination with marked "loading" of the Krebs cycle, followed by a "backing up" of metabolites through the hexose phosphates to glucose. The glucose levels are strikingly different and leave no doubt of the differences between the various types of experiments.

TABLE IV. THE MEAN GLUCOSE LEVELS AT HOURLY INTERVALS

	I. CONTROLS	II. GH	III. GH AND INSULIN	IV. INSULIN
0 ←		50 Mg. %		→
1	224.7	184.8	516.7	637.0
2	246.8	226.2	576.5	673.7
3	353.8	277.3	635.0	685.0
4	390.5	288.6	662.8	832.7
5	486.4	279.0	722.0	881.0
6	479.8	416.0	747.5	883.3
7	462.0	426.6	729.2	926.6
8	532.2	486.8	751.0	907.7
9	544.0	507.6	722.2	910.2
10	497.4	474.7	734.0	938.0
11	487.8	458.0	714.0	968.3
12	509.8	398.4	723.5	960.3

The hepatic protein concentrations indicate a greater production of this particular tissue protein in the presence of GH. This over-all tendency is apparently inhibited by insulin, the mean for the GH-insulin runs being midway between that of the GH group and the controls. Those having insulin alone were indistinguishable from the controls. Taken together with the foregoing plasma protein results, this would appear to indicate that in the GH runs the plasma protein was kept low relative to the levels in the insulin group because of a conversion to tissue protein and/or a direct utilization of the amino acid substrate for tissue protein.* In an organ which is making plasma protein and tissue protein concomitantly, this poses a difficult interpretative problem. In any event, the rise of the plasma proteins to a significantly higher level than in any other group at the conclusion of the experiment at the same time that the hepatic proteins were very significantly higher than those in other experiments is another indication of the superior anabolic activity of GH. Notice also the mixed position of the other groups when these two factors are considered separately; for example, the GH-insulin runs had the lowest total protein level at all times after the second hour but produced somewhat more hepatic protein than did the insulin or control runs. The insulin group had the same final protein concentration as the controls, after the latter had been

*These considerations also explain apparent discrepancies in plasma protein uptake rates and the changes in chemical levels with time.

higher than all others for most of the perfusion time. Finally, the insulin and control group had essentially identical hepatic protein values at a lower figure than the other groups.

The hepatic protein activites show a higher value for the GH group but not to the same extent as chemical protein. For example, insulin and insulin-GH were essentially identical and were not significantly different (see Table III, category L) from GH, in contrast to the chemical analyses. This again would appear to indicate a faster turnover rate for tissue protein under the influence of insulin.

These data indicate that the conclusions of Lotspeich[14] regarding the effectiveness of insulin as an anabolic agent may require re-examination, providing these data can be proved to apply to the intact animal as well as for the isolated liver. The influence of the periphery on these effects is currently under consideration (see below). His conclusions were based on the large decrements in various specific amino acids as determined microbiologically and upon rather good correlation between the various decrements and the composition of muscle. It appears that the biochemical mechanisms of peptidization short of protein synthesis and/or deamination may be such that the tissue protein ratios are preserved in these decrements. Also, such an approach leaves the turnover rates of such protein as may be formed in any particular environment unconsidered.

The greater fall of plasma amino acid concentrations in intact animals when insulin is added to GH which has been reported[20] could also be traceable to this mechanism and would not necessarily represent a true increase in protein anabolism.

Hepatic total lipid and glycogen analyses did not permit clear-cut interpretation because of nonsignificance of all statistical comparisons (see Table III, categories H and I). However, there did appear to be a moderate tendency in both these categories for the groups with insulin to exhibit increases in concentration of both glycogen and lipid. This tendency would appear to be substantiated by the values obtained for liver supernate activity (Table III, category K). It is seen there that the over-all liver supernate activity in the insulin runs is significantly elevated over those in the noninsulin category. The GH and control runs were statistically indistinguishable. The conclusion that the data in this category support a general tendency for the insulin runs to exhibit higher total lipid and glycogen values is based on the assumption that these two groups are the major depots of activity in the liver exclusive of nitrogen-containing compounds. The reason why the GH runs exhibited a relatively high glycogen content is not made clear by the data. It is possible that this merely represents a better preservation of starting glycogen levels (see Table III, category O), although increases in hepatic,[13] tibial,[15] and muscle glycogen[16] have been reported in intact animals receiving GH.

The increase in liver total lipid secondary to insulin is consistent with reported insulin-induced increases of total lipid in intact animals.[17] The absence of lipid increases secondary to GH is also in accord with various experiments utilizing both intact animals[18] and liver slice preparations.[19]

The additive effect which appears to exist with regard to the per cent of counts in the CO_2 traps when GH and insulin are given together can be seen in the data of category M, Table III. These results are made all the more striking because of the virtual identity (numerically as well as statistically) of the GH and insulin groups at a level almost exactly twice that of the controls. Since the effect of combining GH and insulin is additive, we have the rather astonishing situation where the two hormones singly double the control level, and together they quadruple it, doubling their own levels. These data do not help us in localizing the site of action by which these results are effected, nor does it completely rule out the possibility that the effect equivalence is a coincidence of dosage.

It is our interpretation that the increase in CO_2 activity caused by insulin is due to direct deamination of the glycine-2-C^{14}, as well as to the increased turnover of protein amino acids referred to above. As a result of general deamination, carbohydrate residues were employed almost entirely as an energy source rather than fat, resulting in a high residual hepatic fat content and an increased production of $C^{14}O_2$.

It should be noted that an increase in per cent of counts in the CO_2 traps does not necessarily imply an increased production of CO_2 (Table III, category J). It will be seen here that there were no deviations from the control values which were statistically significant. It must be noted that there was a considerable scatter to the individual results when the expired CO_2 was recorded, presumably due to minor leaks of gas from the apparatus in some of the earlier experiments. However, on the basis of our data we are unable to report any significant changes in CO_2 production brought about by changes in hormonal environment. This means that changes in per cent of injected counts in the CO_2 traps must be attributed to deamination and/or increased protein turnover. We have assigned both causes to the insulin increase, since both have been demonstrated to occur. What is the reason for the increase with GH where a lower protein turnover rate occurs? It appears that one must invoke the specific glyconeogenetic properties of glycine[20] coupled with the apparent glycogenetic effect of GH,[15] which has been demonstrated in intact animals, to explain this observation. If the liver, in the presence of GH is stimulated to the production of glycogen, then it is not unreasonable to assume that the glycine present is utilized for its production. This glycogen would then be utilized for energy, resulting in the liberation of $C^{14}O_2$. It is quite likely that the same final reaction (namely, the breakdown of glycogen) is responsible for a portion of the insulin-induced increases, but the pathway by which the glycine-2-C^{14} becomes glycogen is presumably more devious, at least part of it going through a polypeptide and even protein stage prior to deamination. This would be expected from the faster turnover of protein in the insulin group. The reason for the apparent equivalence of the insulin and GH effect is not clear. It would appear fortuitous. If the capacity for the deamination of glycine in the manners indicated is not surpassed, one would expect an additive effect when the two agents are combined.

The final categories (N and O) show the good recoveries of injected radioactivity made and furnish normal values for comparison with the perfusion results.

The normal hepatic content results give the values obtained on a group of twelve rabbit livers, removed after a sudden blow on the head caused the death of the animal. They offer a comparison with the figures reported in the main body of the data and indicate a good approximation to normality of the livers at the end of twelve hours of perfusion. It is interesting to notice that the total protein and glycogen of some of the experimental groups were increased over the values in the normal livers.

Microscopic sections were made of all livers and stained with hematoxylin-eosin and with Schiff's glycogen stain.[21] These showed convincing evidence of cell and organ survival. The remarks which were made in a recent paper[1] describing and illustrating the microscopic anatomy of these livers can be considered to apply to this series with one exception. This group, unlike those illustrated in the publication referred to, were done without cannulation of the common bile duct. This apparently results in the accumulation of edema to a somewhat greater extent than in the group with bile duct cannulation.

SUMMARY

GH has been demonstrated to have an over-all anabolic effect on the protein metabolism of a perfused liver. This effect was achieved by a stimulation of anabolism and a smaller but definite inhibition of catabolism. The effect of GH was superior to that of insulin or of insulin in combination with GH over the twelve-hour period of perfusion, although insulin caused a more rapid initial glycine uptake into plasma protein. The increased catabolic rates in the insulin runs were evidenced by consistently higher urea production throughout the perfusion. Despite increased catabolism, protein anabolism was also stimulated by insulin. This is evidenced by the increased activity in the plasma and hepatic proteins. The turnover rates for both hepatic and plasma protein were more rapid under the influence of insulin. The decreased catabolic rates in the GH runs were shown by consistently lower rates of urea formation in the presence of progressively increasing (to the highest in the series) plasma and hepatic proteins. The urea production was less than with the controls. Insulin resulted in large decreases in the level of chemical alpha amino nitrogen, while the GH group exhibited less of a fall than did the control experiments. This is considered to be the result (primarily) of a shift in equilibrium from the polypeptide to the amino acid pool under the influence of GH as well as of the increase in deamination brought about by insulin (and reflected in the increased glucose and urea levels). The highest hepatic protein specific activities were produced by the livers in the presence of GH. The presence of insulin caused an increase in the hepatic glycogen content (over the controls) and resulted in better maintenance of the hepatic total lipids. This result was confirmed by the greater liver supernate specific activity in the insulin runs. CO_2 production was not significantly altered by these hormones, but insulin

and GH both caused rises in the per cent of the injected activity given off as CO_2. The possible reasons for this are discussed. The effect of GH and insulin on the production of $C^{14}O_2$ are additive.

CONCLUSION

Under these experimental conditions, growth hormone accomplishes an overall anabolic effect with much greater metabolic efficiency and with longer-lasting results than does insulin.

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THE DISTRIBUTION OF LIPID AND PHOSPHOLIPID IN PAPER ELECTROPHORESIS OF NORMAL SERUM LIPOPROTEINS

M. A. Chapin, Ph.D., M.D.
Lewiston, Me.

THE existence of serum lipid in the form of two main fractions, the alpha and beta lipoproteins, has been demonstrated by electrophoresis, ultracentrifugal methods, and chemical separation. In general these methods have indicated that approximately two-thirds of the total serum lipid exists in the beta lipoprotein fraction of normal fasting individuals.

Much evidence has accumulated in recent years indicating that the blood lipids are present in the circulation in combination with protein in the form of lipoproteins. Cohn and his co-workers[1, 2, 3] have shown that all of the lipids of plasma can be recovered in the chemically separated fractions, that the lipids contained in fraction IV + V + VI are combined with alpha$_1$ globulin in the form of alpha lipoproteins, and that those in fraction I + III are bound to beta$_1$ globulin in the form of beta lipoproteins.

From studies in normal individuals, Eder and Russ[4] found about two-thirds of the plasma neutral fat in the beta lipoprotein and the remainder in the alpha fraction. The increment from ingestion of fat, as well as in disease, was found almost entirely in the beta lipoprotein.

The distribution of normal plasma cholesterol in the beta and alpha lipoproteins was found by Oncley[2] to be 75 and 25 per cent, and by Barr,[5] 70 and 30 per cent, respectively. From similar studies, Russ, Eder, and Barr[6] reported that the percentage of cholesterol in the alpha lipoprotein was not related to the level of total cholesterol.

The results of phospholipid studies based on electrophoretic separation, however, have not been in general agreement and have indicated the alpha lipoprotein to contain either most of the phosphatides,[7] 10 to 25 per cent,[8] or, as reported by Kunkel and Slater,[9] slightly less than the beta lipoprotein. Russ, Eder, and Barr,[6] from studies based on chemical separation of the lipoproteins, have reported that in 38 normal subjects the average plasma phospholipid was 253 mg. per cent (range, 188 to 327 mg. per cent) and that the average values for its distribution in the alpha and beta lipoproteins were 107 mg. per cent (range, 76 to 142 mg. per cent) and 116 mg. per cent (range, 61 to 189 mg. per cent), respectively. These results, in spite of the wide range of normal variation and frequent overlapping of values, indicate that the beta lipoprotein contains more phospholipid than the alpha lipoprotein.

From the Department of Medicine, Central Maine General Hospital, Lewiston, Me. This study was supported by a grant from the Maine Heart Association.
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This study of serum phospholipid distribution in normal individuals was prompted by the divergent reports obtained by electrophoretic methods, with the hope that normal variations might be better defined and that pathologic sera might exhibit diagnostic or prognostic variations of significance in disease. While the technique, as employed in the method described below, has the limitations of gross accuracy, it provides an easier method for the over-all study of total lipid and phospholipid distribution in the alpha and beta lipoproteins.

MATERIALS AND METHODS

Sera from normal healthy individuals were selected for this study. This group consisted of 18 young women with range in age of 17 to 30 years and 6 men with age range of 26 to 40 years. Venous blood was drawn from the fasting subject and paper electrophoresis of the serum accomplished within a period of 24 hours, usually within 3 to 6 hours, the sample having been refrigerated until use.

The method of zone electrophoresis was similar to that described by Kunkel and Slater.[9] Whatman 3 mm. filter paper strips, 6 to 12 in number, 1½ in. wide by 9 in. in length, were superimposed on each other, moistened with barbital buffer (pH 8.6, ionic strength 0.1 μ), partially blotted, then inserted between thick glass plates having siliconized surfaces, and the paper ends placed in contact with end buffer compartments holding platinum electrodes. Lead weights giving a pressure of 2 pounds per square inch, were placed on the top glass plate and the sides of the plates sealed with either silicone grease or Scotch tape. Current applied was usually 7 Ma through a variable voltage source of 130 to 150 volts for 30 minutes to allow establishment of equilibrium within the paper. The apparatus was then dismantled and the top glass plate removed. To each paper strip, 2¼ in. from one end, was placed 0.04 ml. serum as a thin band, reaching nearly across the paper. The apparatus was then reassembled and current applied for the usual period of 48 hours, at which time the albumin band front would have migrated 5 in. from the point of application. The paper strips were removed, separated, and dried in an oven at 100° C. for 20 minutes. The low current applied for this longer period of time generally gave more discrete bands and better patterns than a higher current for a shorter time.

The top strip was stained for protein by immersion for 2 minutes in ethanol saturated with mercuric chloride and containing 1 per cent bromphenol blue dye. It was then rinsed with successive portions of 0.5 per cent acetic acid in water until all unstained paper was of normal whiteness. The normal distribution of gamma, beta, $alpha_2$, $alpha_1$ globulins, and of albumin were thus visualized for comparison with the lipid stain of other strips.

In previous experiments it was found that with careful attention to detail, all superimposed strips would stain equally and be in close agreement, thus establishing the validity of lipid estimation in corresponding strips.

Of the remaining strips, two or more were stained directly by immersion in 50 per cent ethanol containing sudan black B dye, according to the method of Swahn.[10] Other strips were placed for 5 minutes each in 3 successive portions of boiling acetone saturated with magnesium chloride, following which they were air-dried and placed in the same sudan staining solution, all strips remaining in the latter overnight for a period of 12 to 14 hours. Excess dye was then removed from the strips by successive immersion in 4 containers of 40 per cent ethanol for 15 minutes each. After air-drying, these strips were ready for measurement with a photoelectric densitometer using a 630 mμ filter. Curves relating optical density and the distance along the paper were established and the areas corresponding to the lipid bands measured by planimeter. For these measurements, some difficulty was encountered in delineating alpha and beta areas, since the interzone trail usually showed small peaks above the baseline. Generally, the lowest point to the baseline just beyond the beta band was selected for the partition, with the beta zone representing the main beta band and the trail from the point of application of the serum; the remaining curve above the baseline from

this partition point represented the alpha zone. One other difficulty concerning establishment of the baseline was overcome by taking densitometer readings well beyond either end of the lipid bands.

Serial determinations have shown the reproducibility of the method to be ± 7 per cent for the directly stained strip and ± 16 per cent for the stained acetone-extracted strip, with the greatest variation present in the smaller alpha group in each instance. Average accuracy was increased by use of multiple strips.

RESULTS

As described by others,[11] the lipids stain in the form of two distinct bands, the larger more intense band corresponding to the beta lipoproteins with the more faintly stained alpha band in the alpha$_1$-albumin area. In all instances, there was a trail from the point of application of the serum to the beta band, with a faint, barely detectable, trail between the two major peaks. These characteristics are more clearly shown by the tracing in Fig. 1, which was obtained from the densitometer readings of a normal serum for protein, total lipid, and phospholipid.

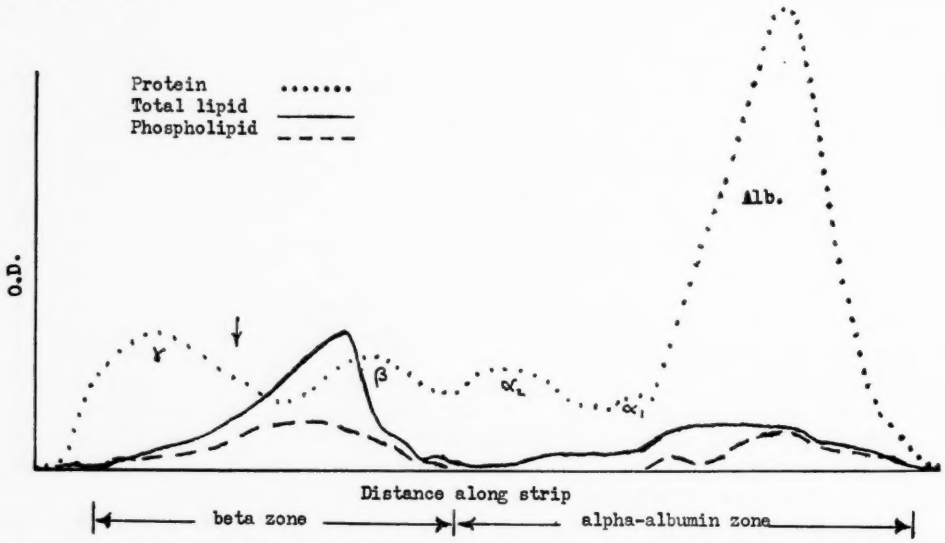

Fig. 1.—Protein, total lipid, and phospholipid curves obtained by paper electrophoresis. The dotted line represents the protein pattern obtained by bromphenol blue dye and densitometer reading. O.D. refers to intensity of dye color. The solid line represents the total lipid and the broken line phospholipid patterns obtained by sudan black B stain of similar paper strips. The point of application of serum to the strips is indicated by the arrow. Separation of lipids into the beta and alpha zones is illustrated. In this illustration, with serum from a normal fasting young female technician, the actual planimeter measurements for the total, beta, and alpha lipids were 11.9, 7.7, and 4.2 sq.cm., and those for the total, beta, and alpha phospholipids were 7.0, 4.8, and 2.2 sq.cm. respectively.

In general, the same findings were obtained from the acetone-extracted strips except that both major bands were less intensely stained, though still visible and in approximately the same areas. As shown in Fig. 1, this stainable residue, representing phospholipid, holds the dye in sufficient amount to permit its gross detection on the paper strip. Although the comparative binding of sudan black B dye by phospholipid and other components of the serum lipids was not determined in this work, the study of this by Swahn[10] indicated their equality in this respect.

Table I lists the results obtained from 23 normal sera. While the average values for distribution of total lipid showed 73 per cent to occur in the beta zone and 27 per cent in the alpha, there was considerable individual variation with a range in the beta lipid, for example, of from 57 to 84 per cent. In general, males tended to have a higher percentage of serum lipids in the beta zone, with a correspondingly lower percentage in the alpha zone. This distribution of the total lipid is in agreement with the results reported by Rosenberg, Young, and Proger,[11] in a similar study with use of sudan IV dye, in which an average of 70.3 per cent of the total lipid was found in the beta zone.

By this method the combined alpha and beta phospholipids were found to average 42 per cent (range, 28 to 60 per cent) of the total lipid, with a 17 and 25 per cent respective composition. This result was in fair agreement with chemical analyses of the same sera which showed phospholipid to average 46 per cent (range, 32 to 63 per cent) of the total lipid.

TABLE I. DISTRIBUTION OF TOTAL LIPID AND PHOSPHOLIPID IN BETA AND ALPHA LIPOPROTEINS*

Total lipid:	73 per cent in beta zone (range 57 to 84)
	27 per cent in alpha zone (range 17 to 43)
Phospholipid:	42 per cent of total lipid (range 28 to 60)
Beta phospholipid:	61 per cent of total phospholipid (range 58 to 82)
	38 per cent of beta lipid (range 30 to 62)
	25 per cent of total lipid (range 20 to 42)
Alpha phospholipid:	39 per cent of total phospholipid (range 21 to 42)
	47 per cent of alpha lipid (range 33 to 72)
	17 per cent of total lipid (range 7 to 19)

*Values are averages obtained from 70 determinations.

With respect to the phospholipid partition, an average of 61 per cent of the total was found in the beta and 39 per cent in the alpha zones. The beta and alpha phospholipids accounted for an average of 38 per cent of the beta and 47 per cent of the alpha total lipid, respectively.

DISCUSSION

While this method of estimation of total lipid and phospholipid, with the separation of these into two main zones by paper electrophoresis and subsequent measurement of dissolved sudan dye with a densitometer and planimetry of the areas thus obtained, is somewhat crude and subject to errors inherent in such histochemical methods, it nevertheless permits an approximate determination of the relative distribution of each lipid component in the alpha and beta lipoprotein zones. In comparison with the complex and time-consuming procedures of chemical fractionation, or with those employing the ultracentrifuge, it offers a relatively simple means of studying the gross lipid and phospholipid variations in health and disease.

The blood lipids are known to have a wide range of normal variation.[6] This study of a small group of apparently healthy individuals gave evidence of this fact with a range of values of 90 to 211 mg. per cent, 162 to 254 mg. per cent and 209 to 584 mg. per cent, for the cholesterol, phospholipid, and total lipid, respectively. This feature of a wide range in normal values is also evi-

dent in the values obtained for total lipid and phospholipid in the beta and alpha lipoproteins, as shown in Table I. Analysis of these results in this small group is therefore impractical.

The main finding of interest in this study was the slight difference in distribution of phospholipid and total lipid between the two lipoprotein zones as separated by paper electrophoresis. While an average of 73 per cent of the total lipid separated with the beta lipoprotein, only 61 per cent of the phospholipid appeared to be present in this fraction. Based on these figures, the total lipid-phospholipid ratios for the beta and alpha lipoproteins were 1.2 and 0.7, respectively. The similarity of these ratios to those obtained by Russ, Eder, and Barr[6] for cholesterol-phospholipid, with averages of 1.35 and 0.52 for the beta and alpha lipoproteins, is apparent.

The fact that phospholipid appears to constitute nearly one-half of the alpha lipid may indicate it to have a different metabolic function from that in the beta lipid. Other observations in favor of this possibility are those of Kunkel and Slater,[9] who noted disappearance of this moiety in certain disease states, and of Maurer and Müller,[7] who incorporated serum phosphatides of rabbits and rats with P_{32}, separated the serum proteins by paper electrophoresis, and determined the radioactivity in each zone. By this technique the bulk of the phosphatides was found to migrate in the albumin zone, indicating that newly formed phosphatides are distributed in the $alpha_1$-albumin lipoprotein complex, as well as indicating the important transport function of the latter with respect to the phosphatides.

From a similar investigation of normal serum lipids, but with use of chemical methods after extraction of paper strips following electrophoresis, Fasoli[8] reported 10 to 25 per cent of the total serum cholesterol and phospholipid to be found in the $alpha_1$-albumin zone. Of the 6 normal sera reported by him, the mean phospholipid in this zone was 24 per cent of the total phospholipid (calculated from data given), which is somewhat lower than the mean obtained in this study.

Kunkel and Slater[9] reported the phospholipid content of the alpha peak to be usually lower than that of the beta compartment. In their method of starch electrophoresis, with use of larger amounts of serum (2 to 5 ml.) and direct analysis of cholesterol and phospholipid in the starch blocks after the run, the cholesterol-phospholipid ratios on a weight basis for alpha and beta liproproteins were found to be 0.41 to 0.62 and 1.17 to 1.36, respectively. Their results were in agreement with those obtained by Russ, Eder, and Barr[6] from an ethanol protein fractionation procedure and direct chemical analysis of the two main fractions, A and C, corresponding to alpha and beta lipoproteins. Russ, Eder, and Barr found the alpha phospholipid to average 44 per cent, with range of 30 to 56 per cent (calculated from data given), of the total phospholipid. The comparable value, or average of 39 per cent, which was found in this study therefore appears to be in agreement with the results of Russ, Eder, and Barr, and those of Kunkel and Slater and would indicate the applicability of this method of study of the phospholipid distribution in the two main lipoprotein fractions.

SUMMARY

A technique employing paper electrophoresis of serum which permits gross estimation of total lipid and phospholipid distribution in the beta and alpha lipoproteins is described and the results obtained in 23 normal subjects is presented.

In this group, 73 per cent of the total lipid and 61 per cent of the phospholipid were found, as general averages, to be contained in the beta lipoproteins. Phospholipid was found to constitute an average of 42 per cent of the total lipid, 38 per cent of the beta, and 47 per cent of the alpha lipids. The lipid-phospholipid ratios for the beta and alpha lipoproteins were found to be 1.2 and 0.7, respectively.

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THE EFFECT OF PLASMA TRANSFUSION AND TREATMENT WITH CORTICOTROPIN ON THE ELECTROPHORETIC PATTERNS IN SERUM AND URINE OF CHILDREN WITH THE NEPHROTIC SYNDROME

GUNNAR B. STICKLER, M.D.,* BERNARD F. MCKENZIE, M.S.,**
KHALIL G. WAKIM, M.D.,*** AND EDMUND C. BURKE, M.D.****
ROCHESTER, MINN.

PREVIOUS studies[1-4] have shown marked differences between serum levels of protein fractions and their concentration in the urine in patients with the nephrotic syndrome. These are: (1) the low concentration of albumin and alpha-1-globulin in the serum and the relatively large amounts of these fractions in the urine; (2) the high concentration of alpha-2-globulin in the serum and the small amounts that are found in the urine; and (3) the relatively small amount of gamma globulin that is found in either the serum or the urine. The measurement of the renal clearance of protein fractions appears to be a satisfactory method to demonstrate this difference quantitatively. Normal urine contains no, or only minute traces of, proteins; therefore the clearance of all proteins in the normal state is essentially zero. In cases of albuminuria, a clearance of albumin of 0.8 indicates that the amount of albumin excreted per minute in the urine is equal to that contained in 0.8 ml. of plasma.

Luetscher[5] has studied the clearance of albumin and alpha-1-globulin in three young adults with the nephrotic syndrome. He determined the relative amount of each of these constituents in plasma and urine by the Tiselius method of electrophoresis at twenty-four-hour intervals. The clearance for albumin ranged from 0.64 to 0.82 ml. per minute, with a mean of 0.39 ml./min./m.^{2} body surface. All three patients received 25 Gm. of purified human serum albumin. The mean albumin clearance increased from 0.39 to 0.66 ml./min./m.^{2}, the globulin clearance remaining the same.

Lauson and associates[6] studied the albumin clearance in 10 nephrotic children treated with corticotropin. Before treatment the albumin clearance ranged from 0.020 to 1.28 ml./min./m.^{2}. After treatment it decreased to 0.0012 to 0.193 ml./min./m.^{2}

McCrory and associates[7, 8] studied the clearance of albumin, alpha-1-globulin, and gamma globulin in five children with the nephrotic syndrome. The

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*Fellow in Pediatrics, Mayo Foundation.

**Section of Biochemistry, Mayo Clinic and Mayo Foundation.

***Section of Physiology, Mayo Clinic and Mayo Foundation.

****Section of Pediatrics, Mayo Clinic and Mayo Foundation.

TABLE I. CLINICAL AND LABORATORY FINDINGS IN FIVE CHILDREN WITH THE NEPHROTIC SYNDROME IN WHOM THE PROTEIN CLEARANCES WERE STUDIED

CASE	AGE (YR.)	WEIGHT (KG.)	DURATION OF DISEASE (MONTHS)	EDEMA (+ TO + + + +)	ASCITES (+ TO + + + +)	BLOOD PRESSURE (MM. HG)	SERUM PROTEIN (GM./100 ML.)	UREA (MG./100 ML.)	CHOLESTEROL (MG./100 ML.)	HEMATURIA (ERYTHROCYTES PER HIGH-POWER FIELD)
1	2⅚	14.5	1½	+ +	0	120/80	2.7	21	327	0
2	4⅓	21.0	1	+ +	+ +	120/70	3.21	46	355	0-8
3	3¼	26	11	+ + + +	+ + + +	100/60	3.08	86	860	Over 31
4	6⅓	31	7	+ + + +	+ +	100/60	1.98	36	634	0-8
5	11⅙	39	5	+ +	+ + +	125/80	1.75	50	794	Over 31

protein fractions were determined by paper electrophoresis in serum and urine. The clearance calculations were based on a single serum specimen and a twenty-four-hour urine volume. They found the clearance in terms of ml./min./m.^{2} to be: albumin, 0.34 to 0.81; alpha-1-globulin, 0.16 to 0.34; gamma globulin, 0.01 to 0.08. They noticed that the alpha-1-globulin clearance approximately paralleled the albumin clearance.

The subjects of this study were five children who suffered from the nephrotic syndrome. Clearances of plasma protein fractions, measured by means of electrophoresis, were studied before and after plasma transfusions. Three of these children were studied further after diuresis was induced by the administration of corticotropin. The clinical findings for these five patients are summarized in Table I. Only those patients were included on whom the diagnosis of nephrotic syndrome was made according to the customary criteria: the presence of proteinuria, hypoproteinemia, hypoalbuminemia, hypercholesteremia, and edema. Hematuria and azotemia may or may not be present. We have added the criterion that there shall be typical changes in the electrophoretic pattern: namely, decrease in the concentrations of albumin, alpha-1-globulin, and gamma globulin, and increase in the concentrations of alpha-2-globulin and beta globulin.

METHOD

At the beginning of the study, an indwelling urethral catheter was inserted to facilitate urine collection. The urine was collected for six to eight periods of three hours' duration, except for one case of marked oliguria in which the periods were lengthened to seven and one-half hours. A blood sample was drawn at the middle of each period.* Intravenous injection of 210 to 250 ml. of plasma per square meter of body surface was instituted at the beginning of the third period and required about three hours. Four of the five patients were then treated for eight days by intramuscular injections of corticotropin to induce diuresis. A total of 150 U.S.P. units per square meter of body surface was given each day in four divided doses. The clearances were determined at three-hour intervals for three additional periods in three patients after the onset of diuresis.

Total serum and urinary proteins were determined by the biuret method. Expediency required that the electrophoretic analysis should be carried out by two methods. The serum proteins were separated on paper by the method of Durrum[9] and quantitated by the procedure of Grassmann, Hannig, and Knedel.[10] Two microliters of serum or concentrated urine were applied evenly across the middle of a 1 by 29 cm. strip of Whatman 2 filter paper and submitted to electrophoresis in the Durrum apparatus for two hours; the current was maintained at 0.5 milliampere. Veronal buffer of ionic strength 0.05 and pH 8.6 was used. At the end of the run, the paper strips were dried at 110° C. for ten minutes. They were stained by the use of amidoschwarz 10B† as described by Grassmann, Hannig and Knedel.[10] Comparison of results obtained by paper electrophoresis with those obtained by the Tiselius procedure indicates that the paper technique can be substituted for the Tiselius procedure.

Because of the large number of determinations to be made during the study on each patient, it was thought desirable to minimize the amount of blood required for experimental purposes; 1 ml. of blood at each interval provided ample serum for the chemical determination and for the separation of the proteins by paper electrophoresis. When this study was

*On the assumption that electrophoretic patterns of serum and urine proteins establish identity, the clearances were calculated by the usual formula, $C = \frac{UV}{P}$, in which U and P designate the amount of the protein component in milligrams per 100 ml. of urine and plasma, respectively, and V is the volume of urine flow in milliliters per minute.

†During the early work on the adaptation of this procedure we used an American preparation of this dye, but the results were not satisfactory. The German-made dye as prepared by Bayerwerke in Leverkusen, Germany, was secured, and thereafter we were able to duplicate closely the results of the German investigators.

instituted, we were unable to obtain paper patterns from urine that were satisfactory for quantitation and we resorted to the Tiselius procedure. At that time we concentrated the urine until the protein concentration was about 6 per cent; 10 ml. portions were dialyzed against three 1 liter portions of Veronal buffer, pH 8.6, molar concentration 0.1. During the dialysis the volume increased considerably; it was finally diluted to 3 volumes with buffer, and at this time the protein concentration was about 2 per cent. Electrophoresis of this solution was performed in the Tiselius apparatus. Before the last case was studied, however, we had devised the following procedure: a measured amount of urine was evaporated under reduced pressure to about ⅕ volume and dialyzed thoroughly against water. It was concentrated again under reduced pressure until the protein concentration was about 8 per

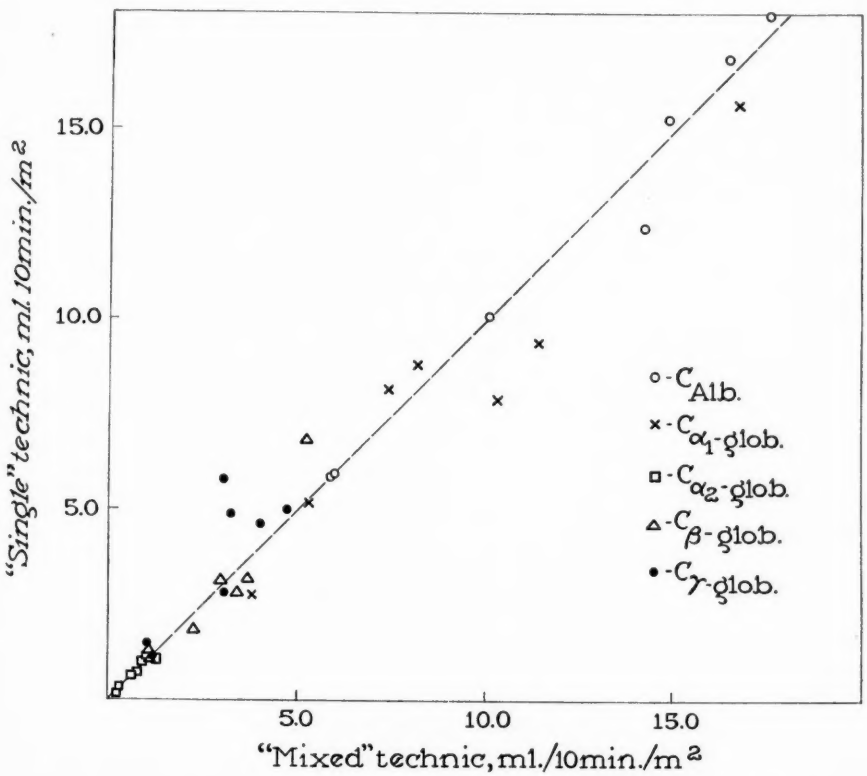

Fig. 1.—Comparison of plasma protein clearances obtained by two different methods. "Single technique": Protein fractions were determined in serum and urine by paper electrophoresis. "Mixed technique": Protein fractions were determined in serum by paper electrophoresis and in the urine by the Tiselius technique.

cent, was equilibrated against Veronal buffer, ionic strength 0.1, pH 8.6, by dialysis, and was finally diluted with the buffer to about 5 per cent protein concentration. Patterns that were satisfactory for quantitation were obtained by means of paper electrophoresis. The solution was further diluted to about 2 per cent protein concentration, and electrophoresis was performed in the Tiselius apparatus. The distribution of the proteins in each urine specimen of Case 5 was determined by both the paper and Tiselius procedures; the clearance values obtained from the two were almost identical. The results are represented in Fig. 1.

RESULTS

The clearance values for all five cases are plotted in Figs. 2, 3, 4, 5, and 6. Several findings should be stressed. The clearance for albumin ranges from

0.17 to 1.31 ml./min./m.² (mean 0.63 ml./min./m.²) and for alpha-1-globulin from 0.11 to 0.77 ml./min./m.² (mean 0.41 ml./min./m.²). In Case 1 the clear-

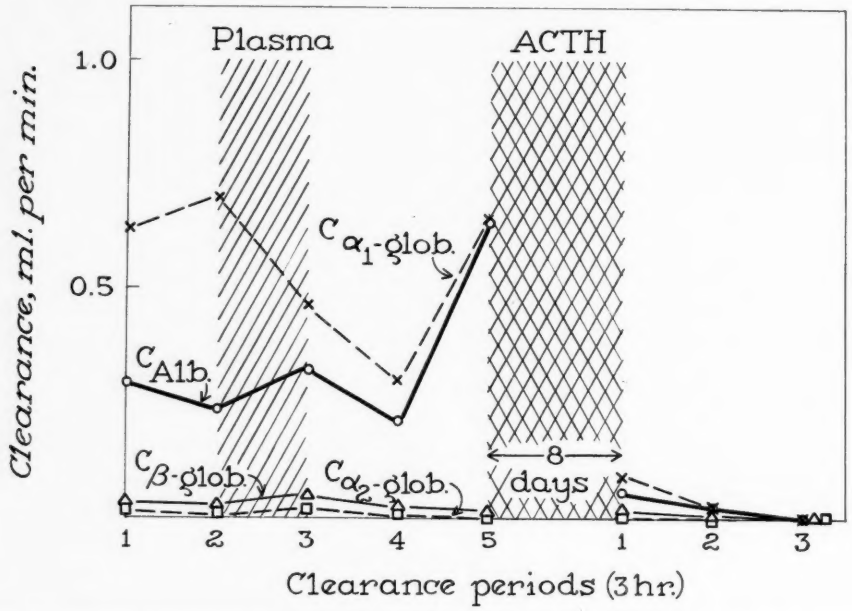

Fig. 2.—Protein clearances in Case 1 before and after plasma transfusion and after corticotropin therapy.

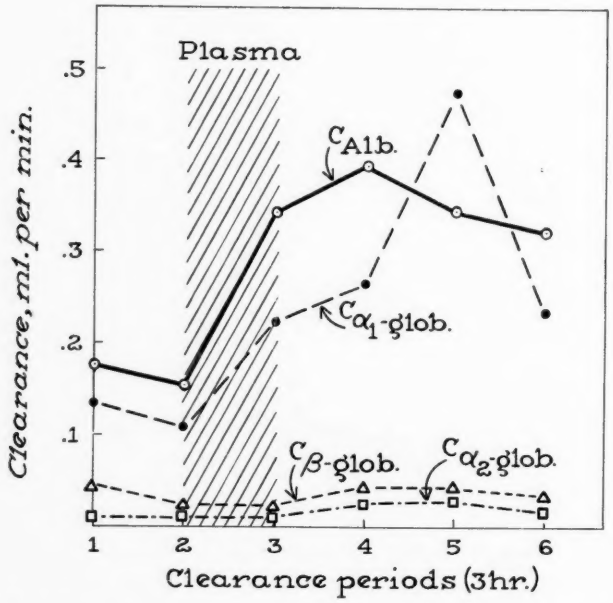

Fig. 3.—Protein clearances in Case 2 before and after plasma transfusion.

ance values for alpha-1-globulin were higher than those for albumin; in general, the clearance values for these two proteins parallel each other fairly well and

are consistently higher than those of the remaining serum proteins. In only two cases could the clearance of gamma globulin be determined. In these cases an appreciable amount of this protein was found in the urine. The inability to determine the clearance of gamma globulin in the other cases was in part due to the difficulty of measuring small amounts of gamma globulin by the Tiselius procedure. Hayles, Stickler, and McKenzie[11] noted that small

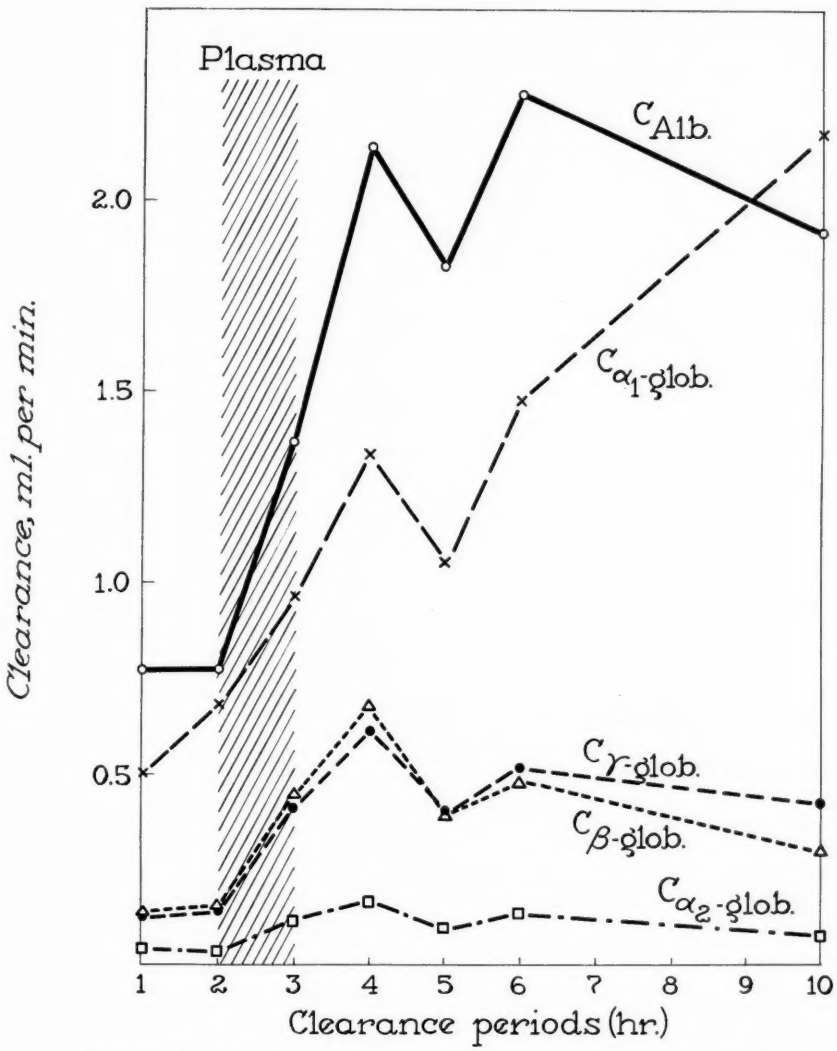

Fig. 4.—Protein clearances in Case 3 before and after plasma transfusion.

amounts of gamma globulin may be detected by paper electrophoresis but escape detection in the Tiselius apparatus. Wall and Saslaw[12] stated that gamma globulin may appear to be completely missing in the Tiselius pattern but may be as high as 1 to 1.5 per cent of the total protein by paper electrophoresis. Cases 1, 2, and 4 showed a marked rise in clearance values for albumin and alpha-1-globulin after plasma transfusion; Cases 3 and 5 showed an increase in the

clearance of all protein fractions. It was noted that the latter two cases showed microscopic grade 3 hematuria (31 or more erythrocytes per high-power field). After the onset of diuresis, the clearance of all proteins decreased markedly.

Five hundred milliliters of plasma was given in Case 5 during diuresis, and the clearance values remained low in the succeeding twelve hours of study (Fig. 6).

Fig. 7 shows the mean serum concentration of the protein fractions in all five cases. It was noted that the alterations in concentration were not very marked even after the administration of plasma and of corticotropin.

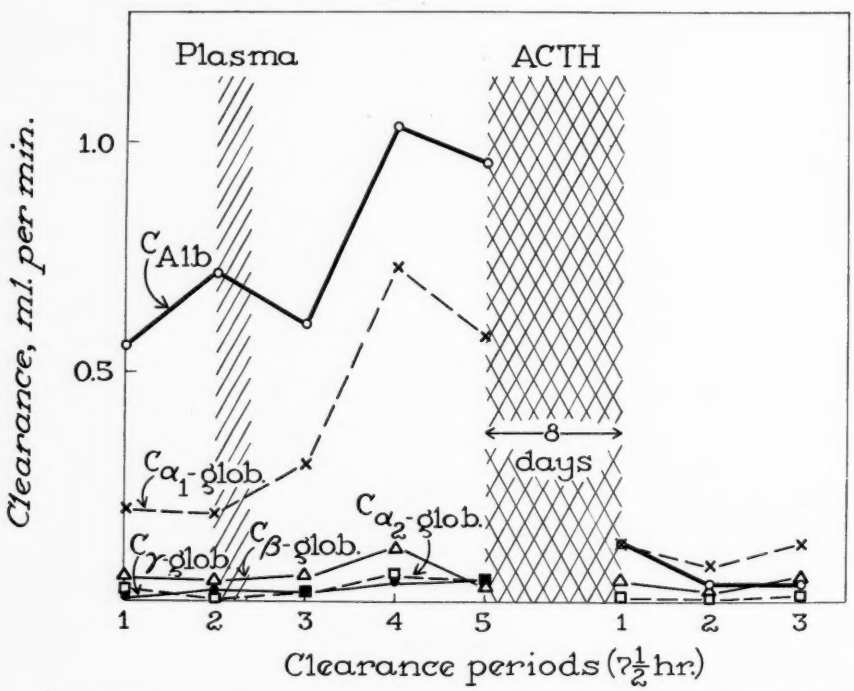

Fig. 5.—Protein clearances in Case 4 before and after plasma transfusion and after corticotropin therapy.

The percentage and absolute amount of the protein fractions in each unit of plasma for transfusion were determined, and the amounts of these fractions which were excreted in the urine at intervals of three, six and nine hours were calculated (Table II). After nine hours, amounts of albumin and alpha-1-

TABLE II. PERCENTAGE OF PROTEIN FRACTIONS EXCRETED FROM THE TRANSFUSED PLASMA IN 3, 6, AND 9 HOURS AFTER TERMINATION OF THE TRANSFUSION

PROTEIN FRACTION	PERCENTAGE EXCRETED		
	3 HOURS	6 HOURS	9 HOURS
Albumin	6	18	29
Alpha-1-globulin	7	19	30
Alpha-2-globulin	5	11	16
Beta globulin	3	5	14

globulin equivalent to 29 and 30 per cent, respectively, of these fractions that were added by transfusion were lost in the urine; the loss of the other fractions was much lower.

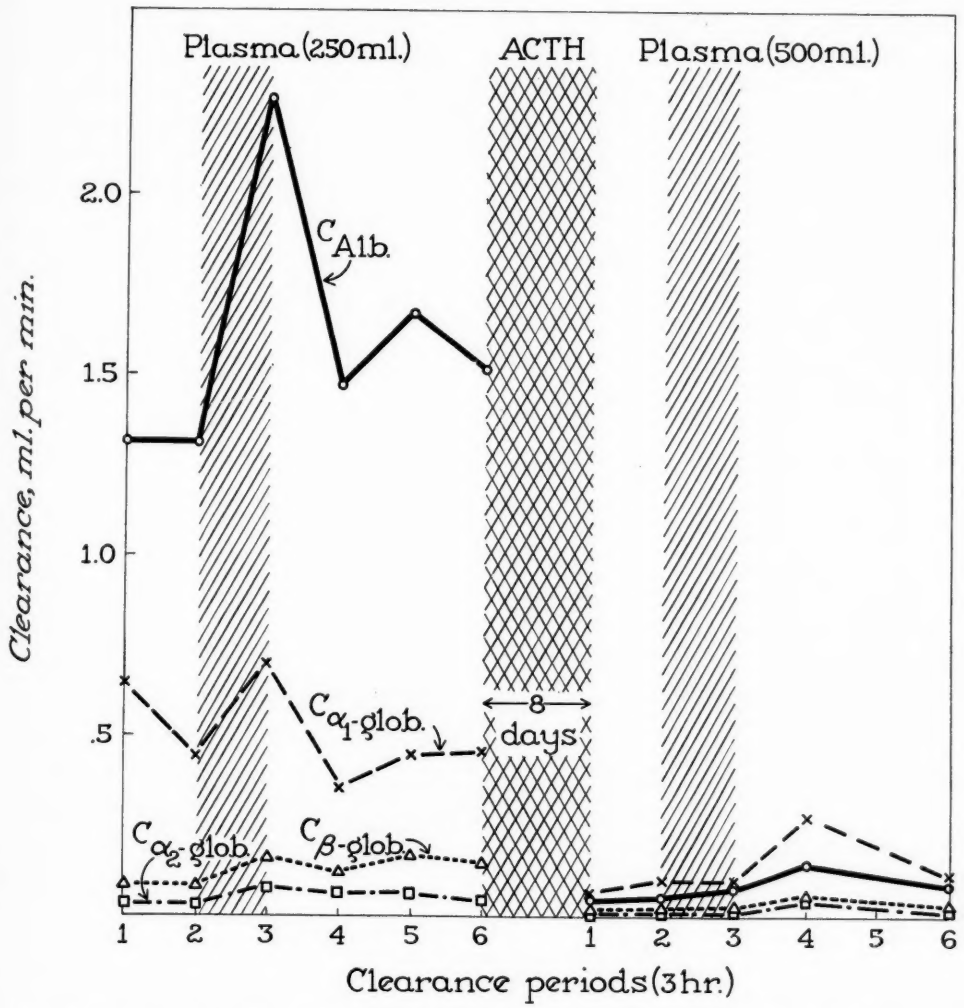

Fig. 6.—Protein clearances in Case 5 before and after plasma transfusion and after corticotropin therapy, which was followed by another plasma transfusion.

COMMENT

The methods employed in this study allow definite statements only about the clearance of protein fractions with identical electrophoretic mobility in serum and urine. Criticism might be expected on account of the fact that in four of the five cases, two different electrophoretic procedures were used for serum and urine. But in our experience, the Tiselius technique and the paper technique of electrophoresis correlate well enough to be used simultaneously. The results we obtained in one case, in which clearances were determined by

measuring the concentration of protein fractions in serum and urine by two different procedures as well as by a single technique, have justified this assumption (Fig. 1).

Lauson and Chinard,[13] and Chinard and associates[14] have presented convincing evidence that proteinuria in the nephrotic syndrome is due to increased permeability of the glomeruli. McCrory and associates[8] have pointed out that proteins of smaller dimensions probably pass through this abnormally permeable membrane more readily than larger molecules. This would explain the high clearance of albumin (molecular weight 69,000), and it may be assumed that the molecular weight and electrophoretic mobility of the albumin fractions

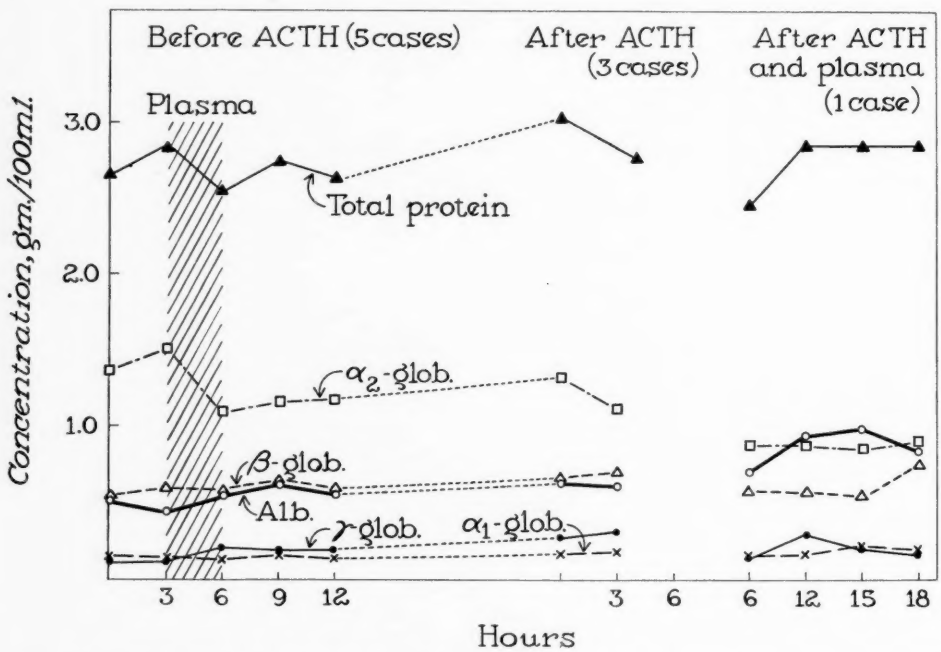

Fig. 7.—Mean serum-protein levels before and after plasma transfusion and after corticotropin therapy (in one case following a second plasma transfusion).

are the same in serum and urine. The high clearance of alpha-1-globulin is more difficult to explain, since the molecular weight of the ethanol-prepared alpha-1-lipoprotein in the serum of normal persons is thought to be close to 200,000.[15] It should be emphasized that the electrophoretic alpha-1-globulin fraction differs from the ethanol-prepared alpha-1-lipoprotein. Smaller molecules with the electrophoretic mobility of alpha-1-globulin may be the ones excreted in the urine. In recent preliminary studies, Slater[16] has shown that the molecular weight of alpha globulin in the urine of a patient with the nephrotic syndrome was 45,000, and from his illustrations we are inclined to believe that it is alpha-1-globulin. The low clearance of the other globulins is probably due to their higher molecular weight. Only simultaneous study of the molecular weight of the electrophoretic protein fractions in serum and urine could give definite information in this regard.

The increase in the clearances of albumin and alpha-1-globulin after transfusion of plasma may be explained by an increase in plasma flow, but we have no data to support this thesis. It is interesting to note the high clearance of albumin in the two cases of marked hematuria; in these two cases even the globulin clearances increased after the plasma transfusion. The mechanism of hematuria in the nephrotic syndrome is unknown; but one could speculate that, owing to rupture of some of the glomerular capillaries (Bell[17]), more erythrocytes and globulins of higher molecular weight will leave the circulation.

The reduction in the clearances after the administration of corticotropin is marked. Heymann and associates[18] have postulated recently that this is due to a decrease in the permeability of the glomerulus to protein. The fact that the concentration of the protein fractions in the serum does not increase significantly and the failure to recover these fractions in the urine after transfusion of plasma could be due to at least three factors: (1) metabolization of some of the proteins, (2) an increase in the circulating plasma volume and (3) escape of some proteins into the extravascular space (Gitlin and Janeway[19]).*

SUMMARY

The renal clearances of the plasma protein fractions were studied in five children suffering from the nephrotic syndrome both before and after the administration of plasma and of corticotropin. There was a marked increase in the clearance of albumin and alpha-1-globulin during the edematous phase after administration of plasma. In only two cases with marked hematuria was there an increase in the clearance of the other globulins before diuresis. In three cases all clearance values decreased considerably after diuresis was induced by administration of corticotropin, and in one case, clearance values did not increase after transfusion of plasma. This suggests that plasma transfusion may be beneficial after diuresis occurs in the patients treated with corticotropin.

These findings in the nephrotic syndrome might be explained on the basis of increased glomerular permeability to proteins of lower molecular weight. After the administration of corticotropin, the decreased clearance values seem to suggest a decreased glomerular permeability.

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*In one case, it was shown that the transfusion of plasma during diuresis, induced by corticotropin, failed to raise the protein clearance. If this could be demonstrated in more patients, it might indicate that the administration of plasma to such patients during diuresis helps to restore the plasma proteins more rapidly.

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STUDIES OF SERUM MUCOPROTEIN (SEROMUCOID)

I. A Turbidimetric Method

J. de la Huerga, M.D., Alvin Dubin, M.S., Daniel S. Kushner, M.D.,
H. A. Dyniewicz, Ph.C., and Hans Popper, M.D.
Chicago, Ill.

INTRODUCTION

THE clinical significance of the determination of the various constituents of serum glycoproteins has been emphasized by the work of Winzler and Greenspan.[1-8] While all serum proteins contain carbohydrates, the fraction nonprecipitable by perchloric acid has a larger content of carbohydrate and hexosamine.[2, 9] In this fraction, originally designated as mucoprotein, the carbohydrates are strongly bound to the protein component. More recently the term "seromucoid"[10, 11] has been reapplied to distinguish this material from other more easily hydrolyzed carbohydrate-rich proteins.[12]

The methods in common use for the measurement of mucoproteins are based on three steps[2]: (1) precipitation of other proteins by perchloric acid, (2) isolation of the mucoproteins in the filtrate by phosphotungstic acid, and (3) determination of the protein by either its "tyrosine" content[2] or by the biuret reaction.[13] The method to be described combines steps 2 and 3 by turbidimetric estimation of the entire mucoprotein precipitate.

MATERIALS AND METHODS

Reagents.—

1. 0.85 Per cent sodium chloride
2. 1.8 N Perchloric acid
3. 5 Per cent phosphotungstic acid in 2 N hydrochloric acid

Procedure.—Transfer 2 ml. of serum into a 125 ml. Erlenmeyer flask and add 18 ml. of 0.85 per cent sodium chloride solution. Mix by swirling, and while rotating the flask, add dropwise 10 ml. of 1.8 N perchloric acid solution. Allow to stand at room temperature for 15 minutes and filter through 9 cm. Whatman No. 50 filter paper. The filtrate obtained must be water clear. Transfer 15 ml. of the filtrate into an 18 by 50 mm. test tube, calibrated at 6 ml. To this add 3 ml. of the phosphotungstic acid reagent, close with a No. 1 rubber stopper, and invert 3 times. Allow to stand at room temperature for 20 minutes. Centrifuge at about 500 r.p.m. for 5 minutes. Siphon off the supernatant fluid to the 6 ml. mark. Stopper the tube again and invert 3 times. Transfer the whole volume to a cuvette, and read in a Coleman Universal spectrophotometer at 650 mμ, using water as a blank.*

Standardization.—Purified serum mucoprotein (MP-1), prepared by the method of Weimer and associates[9] and treated as described in the procedure, was found to give a linear

From the Hektoen Institute for Medical Research of the Cook County Hospital, The Departments of Pathology and Medicine, Northwestern University Medical School, and The Chicago State Tuberculosis Sanitarium, Chicago, Ill.

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*A cuvette carrier adapted to a 5 ml. volume can be obtained from Wilkins-Anderson & Company, Chicago, Ill.

relationship between concentration and optical density and served as a standard for the values reported below. To provide more easily available turbidimetric standards, various turbid solutions, including the barium sulfate standard,[14] were tried. The latter was discarded because the ratio of the optical density of barium sulfate to that of the mucoprotein precipitate varied with the wave length (Fig. 1). The method finally chosen was that whereby turbidity was obtained by precipitating with phosphotungstic acid Armour's bovine serum albumin, which is a homogeneous fraction, electrophoretically pure and commercially available. To different dilutions of this preparation, containing from 50 to 500 μg of protein nitrogen in 5 ml. of aqueous solution, was added 1 ml. of the phosphotungstic reagent. After standing for one-half hour, the sedimented precipitate was redistributed by inversion of the tubes 3 times, and readings were recorded at various wave lengths. The spectral curve was identical with that of precipitated mucoproteins (Fig. 1). Serums containing 250 mg. per cent of mucoprotein gave the same turbidity through the whole visible spectrum as did an aqueous solution containing 500 μg of bovine albumin nitrogen in 5 ml. This permits

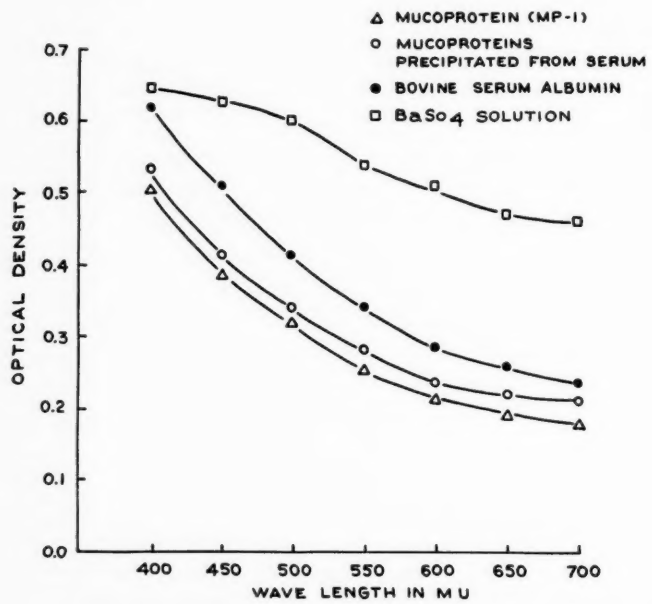

Fig. 1.—Variance of absorption of barium sulfate suspension from that of mucoprotein and albumin suspensions.

standardization with any spectrophotometer or filter photometer. The ratio between the albumin standard and the precipitated mucoproteins is the same at various wave lengths of the Coleman Universal spectrophotometer as in the Evelyn colorimeter with use of filters 650, 520, and 440 mμ (Fig. 1). The 650 mμ wave length was selected for the Coleman Universal spectrophotometer, but for other instruments which do not have as high optical absorption of turbidity, a lower wave length is recommended. For example, in the Coleman Junior spectrophotometer, readings at 500 mμ are of similar magnitude to those obtained with the Coleman Universal spectrophotometer model at 650 mμ.

Effect of Variation of Procedure.—Varying the temperature from 20° C. to 32° C. throughout the procedure did not alter the turbidity. The perchloric acid filtrates were allowed to stand from 20 to 60 minutes after addition of the phosphotungstic reagent before centrifugation without appreciable variation in the readings. Variation of the speed of centrifugation from 500 to 1,500 r.p.m. for periods from 5 to 15 minutes did not influence the results. However, if centrifugation was carried out at a higher speed or

for a longer period of time, the mucoprotein particles aggregated with resulting variations in the readings. Replication of the procedure on 4 successive days in frozen serum gave reproducible results. Similar reproducibility was obtained in perchloric acid filtrates refrigerated at +5° C. for 4 days.

Normal Values.—Serum mucoprotein levels were determined with the method described in 30 normal young women and 40 men (nurses, technicians, and medical students). All sera were obtained in the early morning from subjects in the fasting state. The values for women (95 per cent of subjects) ranged between 60 and 100 mg. per cent, with a

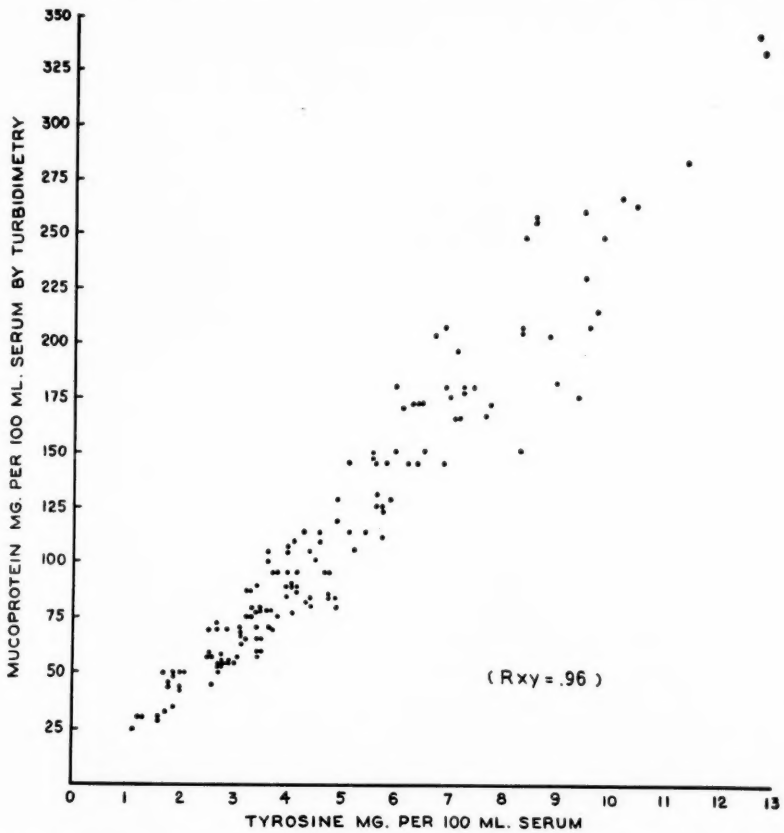

Fig. 2.—Comparison of serum mucoproteins determined by the tyrosine method and by the turbidimetric method in 184 individuals.

mean and standard deviation of 77.1± 14 mg. per cent. The men (95 per cent of subjects) showed a range from 55 to 105 mg. per cent, with a mean and standard deviation of 81.2 ± 18 mg. per cent.

Comparison of the Tyrosine Colorimetric Method With the Turbidimetric Method.—Serum mucoproteins were determined by both the tyrosine[2] and turbidimetric methods in 53 normal adults and 131 patients hospitalized for various diseases (Fig. 2). The mean tyrosine value in normal individuals was 2.9 mg. per cent. Comparison of values obtained by the two methods showed a correlation coefficient (Rxy) of 0.96, indicating excellent agreement in both the normal and abnormal range of values. However, in individual cases, discrepancies in the results by both methods occurred.

Micro Method.—A modification of the above described procedure was devised to utilize only 0.5 ml. of serum.

Transfer 0.5 ml. of serum to a 15 × 125 mm. test tube. Add 4.5 ml. of 0.85 per cent sodium chloride solution and 2.5 ml. of 1.8 N perchloric acid. Mix by inversion and allow to stand 15 minutes. Filter through 7 cm. Whatman No. 50 filter paper. The filtrate must be water clear. Transfer 5 ml. of filtrate to a 19 mm. cuvette and add 1 ml. of the phosphotungstic acid solution. Invert but do not shake. Allow to stand for 30 minutes. Read at 400 mμ (Coleman Universal spectrophotometer), using water as a blank.

Parallel mucoprotein determinations in 600 sera with both methods described gave mean values with the micro method which were 10 per cent higher than those obtained with the macro technique (Fig. 3).

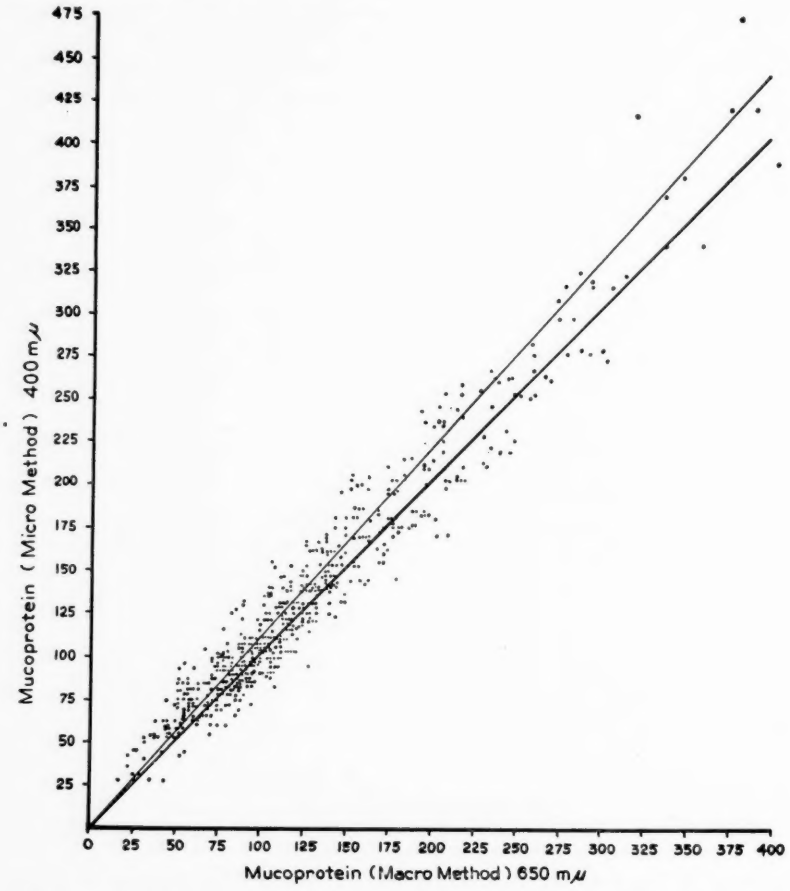

Fig. 3.—Correlation between serum mucoprotein concentration determined in 600 sera with techniques employing 2 ml. and 0.5 ml. of serum. The light upper line represents the correlation between the two methods. The heavy lower line is the line of theoretical correlation.

With use of this modification of the procedure, an aqueous solution containing 200 μg of bovine albumin nitrogen in 5 ml. gave the same turbidity as serum containing 252 mg. per cent of mucoprotein, when standardized with use of purified mucoprotein.

DISCUSSION

The most critical factor in achieving reproducibility of the method was the manner of preparing the perchloric acid filtrate, especially with regard

to the serum dilution. The method described follows the procedure of Weimer and Moshin[15] for the precipitation of serum proteins.

The turbidimetric method reflects the entire mucoprotein complex, while other methods measure components. The method is simple, reproducible, and lends itself to large-scale determinations.

Serum mucoprotein levels determined as tyrosine range in normal individuals between 2 and 4 mg. per cent, with a mean of 2.7 ± 0.05 mg. per cent.[3] Mucoprotein values determined as tyrosine may be converted to protein values by dividing by the tyrosine content of mucoprotein. The tyrosine content of our mucoprotein standard was 3.65 per cent, in agreement with that of 3.6 per cent found by Weimer.[9] The mean value in normal serum obtained in this study by the tyrosine method was 2.9 mg. per cent. This would give a mean mucoprotein value of 79.5 mg. per cent, calculated from the tyrosine values, thus corresponding to the observed over-all mean value of 79.4 mg. per cent obtained turbidimetrically.

With the biuret method, normal values have been reported to range between 40 and 90 mg. per cent by some investigators,[13] while Greenspan reported a normal range of 48 to 75 mg. per cent (mean 59.2 mg. per cent) for men and 40 to 70 mg. per cent (mean 52.9 mg. per cent) for women.[7] With bovine serum albumin as standard, the protein content of our mucoprotein preparation was 64.5 per cent, consistent with that observed by Winzler[2] of 58 per cent. Thus a mean value in normal serum of 79.4 mg. per cent would correspond to a value of 51.2 mg. per cent determined by the biuret method. These comparisons must be considered approximations, however, since they are based on a comparison with a purified mucoprotein (MP-1), whereas the mucoprotein fraction determined is actually heterogeneous.

An alternative turbidimetric method adaptable to small volumes of serum is described for use in children or experimental animals. However, in view of the relatively small degree of turbidity obtained, this method can only be applied if sensitive instruments such as Coleman Universal or Bausch and Lomb spectrophotometers are available.

SUMMARY

A simple turbidimetric method for determination of serum mucoprotein (seromucoid), applicable to routine use in the clinical laboratory, is described. It measures the entire mucoprotein complex, rather than its constituents. The mean level in normal women was 77.1 ± 14 mg. per cent and in men 81.2 ± 18 mg. per cent. Excellent agreement was obtained with the tyrosine method in the abnormal as well as in the normal range of values.

We are grateful to Dr. Richard J. Winzler for a generous supply of serum mucoprotein (MP-1) and for advice and encouragement in these studies and in preparation of the manuscript.

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STUDIES OF SERUM MUCOPROTEIN (SEROMUCOID)

II. Physiologic Variations and Response to Stress

Daniel S. Kushner, M.D., Klaas Honig, M.D., Alvin Dubin, M.S.,
H. A. Dyniewicz, Ph.C., David Bronsky, M.D., J. de la Huerga, M.D.,
and Hans Popper, M.D.
Chicago, Ill.

INTRODUCTION

THE concentration of serum mucoprotein (seromucoid) is elevated in many unrelated physiologic and pathologic states. An excellent review of these and related glycoproteins has been recently made by Winzler.[1] Essentially similar substances have been studied under the designations of "index of polypeptidemia," blood proteose, seroglycoid, mucopolysaccharide, mucoprotein, and seromucoid.[2] The wide variety of unrelated disease states and experimental conditions in which serum mucoprotein is elevated has led to the suggestion that mucoprotein elevation may be a response to acute and chronic stress.[3-5] The present study was undertaken to define the degree of physiologic variation of serum mucoprotein concentration and the effect of various stresses, independent of disease processes, in order to evaluate separately the physiologic implications and diagnostic significance of alterations in disease.

MATERIAL AND METHODS

Serum mucoprotein was measured by the turbidimetric method previously described.[6] Subjects were patients hospitalized at Cook County Hospital with stable illness and with no apparent acute infectious, inflammatory, neoplastic, or hepatic disease, with normal endocrine function, and, in most instances, with normal serum mucoprotein levels. Subjects were selected for study only if several successive mucoprotein levels varied less than 10 per cent.

The study was concerned with (1) the constancy of levels in the fasting state on successive days, (2) the effect of age, (3) the diurnal variations, (4) the effect of meals, (5) stress situations produced by injection of histamine, bacterial pyrogen (heat-killed typhoid bacilli), adrenocorticotrophic hormone (ACTH), parathyroid extract,* and by major surgical operations.

The method was previously found to be reproducible within ±5 per cent. Therefore for the purpose of this study, only variations of greater than ±10 per cent were considered significant.

RESULTS

Variation of the Serum Mucoprotein Level on Successive Days.—The fasting serum mucoprotein concentration of 18 hospitalized patients in the basal

From the Hektoen Institute for Medical Research of the Cook County Hospital; the Departments of Pathology and Medicine, Northwestern University Medical School; and the Oak Forest Infirmary, Chicago, Ill.

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*The parathyroid extract used in those studies was generously supplied by Eli Lilly and Company, Indianapolis, Ind.

state on four successive days did not vary by more than 5 per cent (Fig. 1). Similar stability was subsequently observed in many other patients, at various levels within the normal range.

Effect of Age.—Serum mucoprotein was determined monthly for a period of six months in 26 individuals (21 men, 5 women) ranging in age from 71 to 94 years. The mean of all determinations was 106 ± 21 mg. per cent, with the range of 95 per cent of values falling between 75 and 130 mg. per cent. The mean value previously found in young healthy adults was 79.4 mg. per cent.[6]

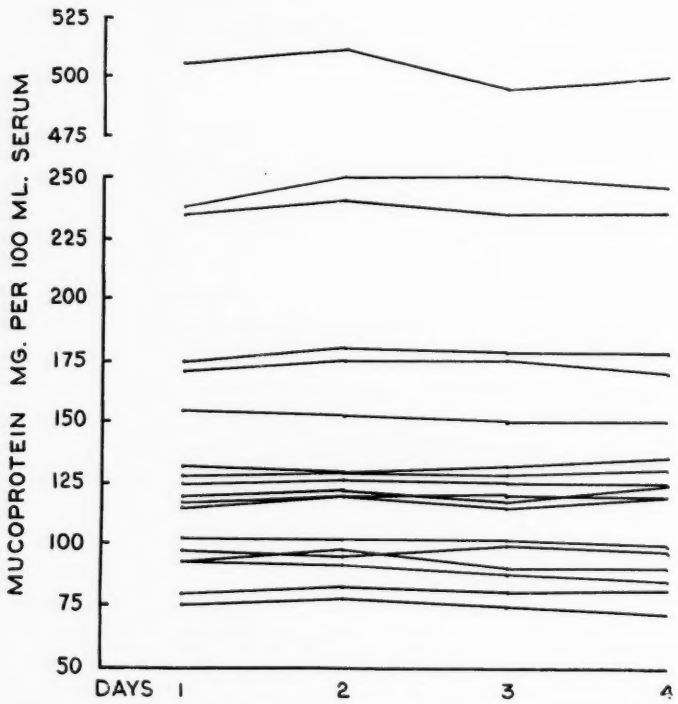

Fig. 1.—Constancy of fasting serum mucoprotein levels determined in 18 patients on four successive days.

Diurnal Variation.—An increase in excess of 10 per cent of the fasting mucoprotein level was observed within an eight-hour period in 11 of 20 subjects. Meals were permitted at the usual hours. The trend of values in successive samples was progressively upward and the highest mucoprotein concentration was consistently encountered in the late afternoon specimens. The greatest increment was 59 per cent of the fasting value (Table I). The mucoprotein concentration determined the following morning showed a return to previous fasting levels. In no instance was a significant decrease in level encountered.

Effect of Meals.—In 12 patients, serum mucoprotein concentration determined just prior to and following the noon meal did not vary more than 10 per cent (Table II). In no instance did the level significantly increase.

Stress.—

Effect of histamine: Histamine phosphate (1 mg. of histamine base) was given to three subjects in continuous intravenous infusion over a period of

TABLE I. DIURNAL VARIATION OF SERUM MUCOPROTEIN VALUES

SUBJECT	DAY	FASTING LEVEL (MG./100 ML.)	MAXIMUM LEVEL (MG./100 ML.)	% VARIATION
L. W.	1	160	167	4.4
	2	155	178	15.0
E. J.	1	77	96	25.0
	2	88	102	16.0
J. S.	1	122	130	6.6
	2	130	133	3.9
L. J.	1	63	70	11.0
	2	64	85	33.0
J. H.	1	183	244	33.0
	2	220	306	39.0
M. D.	1	85	98	15.0
	2	108	108	0.0
M. H.	1	75	104	39.0
	2	86	106	23.0
B. S.	1	128	203	59.0
R. C.	1	116	114	1.7
	2	98	100	2.0
	3	100	104	4.0
J. M.		165	175	6.1
A. M.		118	120	1.7
G. K.		128	112	-13.0
B. M.	1	98	104	6.1
	2	100	108	8.0
D. J.	1	94	100	6.4
	2	90	94	4.4
	3	88	102	16.0
C. F.	1	102	96	-5.9
	2	100	110	10.0
	3	100	98	-2.0
D. A.		100	148	48.0
I. C.		96	100	4.2
R. J.		70	77	10.0
L. C.		102	128	26.0
F. McG.		104	120	16.0

TABLE II. EFFECT OF MEALS ON SERUM MUCOPROTEIN LEVEL

SUBJECT	BEFORE LUNCH	AFTER LUNCH	PERCENTILE CHANGE
E. R.	61	60	1.6
A. D.	137	135	1.5
J. Cu.	130	135	3.8
E. F.	100	106	6.0
A. S.	126	128	1.6
J. Ca.	135	141	4.4
J. R.	60	66	10.0
J. W.	155	160	3.2
R. T.	83	88	6.0
L. Pa.	90	102	13.0
L. Pr.	203	211	2.5
H. M.	137	137	0.0

five hours on three successive days. Serum mucoprotein levels before, during, and upon completion of each infusion over a six-day period ranged from 70 to 94 mg. per cent, 96 to 120 mg. per cent, and 120 to 155 mg. per cent, respec-

tively. In the three subjects, the difference between the mean control and the maximum mucoprotein concentration encountered during the study period was 25 per cent, 16 per cent, and 29 per cent, respectively.

Effect of bacterial pyrogen: Fever was induced in two subjects by multiple intravenous injections of heat-killed typhoid bacilli in graduated dosage at intervals of several days. Serum mucoprotein concentration was determined in the fasting state, at height of fever, and in late afternoon. In both subjects each injection was followed by fever in excess of 103° F. and by

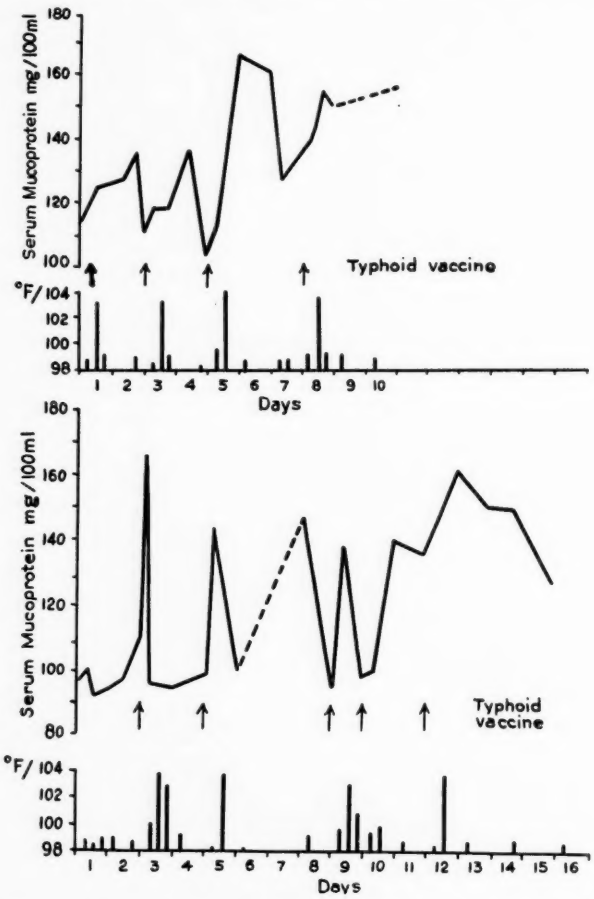

Fig. 2.—Serum mucoprotein and temperature response to intravenous injection of typhoid vaccine.

elevation of mucoprotein. The difference between the mean control value and maximum mucoprotein level was 45 per cent and 74 per cent respectively. After several injections, elevation of mucoprotein persisted, 36 per cent and 53 per cent above baseline values, despite absence of fever (Fig. 2).

Effect of ACTH: In preliminary observation, the mucoprotein level in six individuals varied less than 10 per cent in at least three determinations within a 24-hour period. During the study, mucoprotein was determined be-

fore, during, and upon completion of ACTH infusion. In the first subject, a single infusion of 30 units given over eight hours did not significantly alter mucoprotein levels during the subsequent 72-hour period. In two others nonresponsive to a single infusion, a second infusion of 30 units given 48 hours later was followed by a rise of 30 to 60 mg. per cent, respectively, from a baseline of 100 mg. per cent. Two other subjects were then given infusions of 30 units on successive days. Again the initial injection resulted in little change, but at the completion of the second infusion the mucoprotein level rose 55 mg. per cent from a baseline of 100 mg. per cent, returning to normal within 72 hours. A sixth subject was given two successive daily infusions of 40 units; each was accompanied by a prompt rise in levels of 50 mg. from an initial value of 110 mg. per cent. The observed 50 per cent rise of mucoprotein in four of the subjects was considered significant in view of the established

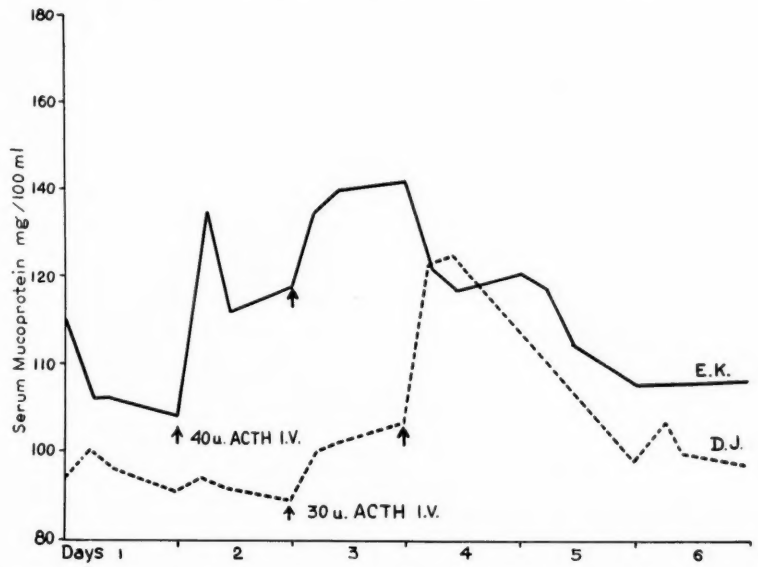

Fig. 3.—Mucoprotein response to ACTH infusion at dosage levels of 30 and 40 units.

constancy of levels in these subjects. The response of two subjects at dosages of 30 units and 40 units is shown in Fig. 3.

Effect of surgical operation: Daily fasting mucoprotein levels were determined pre- and postoperatively in 28 patients undergoing major surgical procedures (Table III). In every instance, a significant elevation occurred irrespective of whether the initial values were low, normal, or high, and of the type and extent of operative procedure. In 10 patients, the mucoprotein rose significantly on the first postoperative day; the increment ranged from 20 to 114 per cent of the initial values. The peak elevation occurred in most instances on the third or fourth postoperative day, the maximum increment ranging from 26 to 164 per cent of the initial levels. Mucoprotein returned to the normal or preoperative range only in the third postoperative week; the earliest return to normal occurred on the eighth day.

Effect of parathyroid extract: In five subjects, the intravenous injection of parathyroid extract (200 to 500 units) was followed by a prompt rise of serum mucoprotein concentration. The increments in two euparathyroid subjects given 200 units of extract were 38 per cent and 74 per cent, respectively. The remaining three subjects were given 500 units and had increments of 48 per cent, 77 per cent, and 140 per cent. A significant rise occurred within two hours, with the peak at five hours in four of the five subjects; in one the maximum level developed within 24 hours. The greatest response occurred in a patient in the postoperative state, with postthyroidectomy hypoparathy-

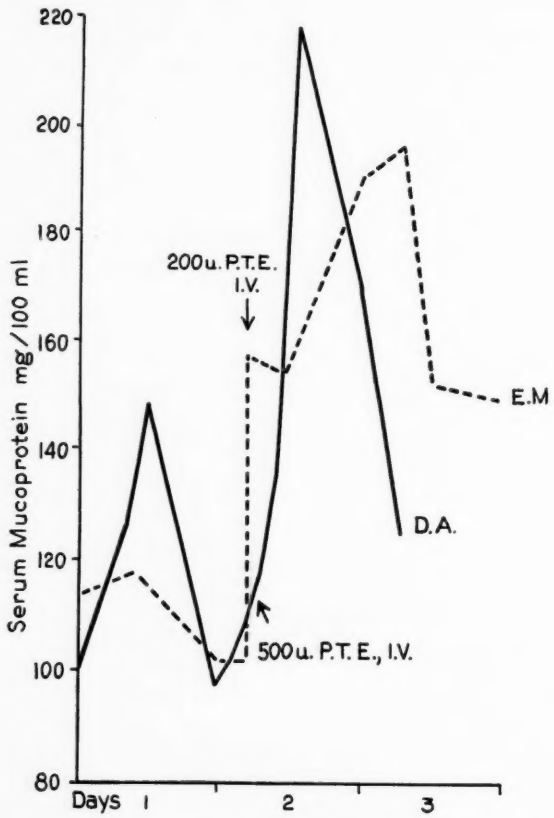

Fig. 4.—Mucoprotein response to injection of parathyroid extract at dosage levels of 200 and 500 units.

roid tetany. The mucoprotein level rose from 197 to 473 mg. per cent within two hours following injection and fell to 162 mg. per cent within five hours. It is therefore assumed that the postoperative state alone could not account for the marked and transient mucoprotein change. The increment of 48 per cent following parathyroid extract on the sixth postoperative day occurred in a patient with mild tetany following resection of a parathyroid adenoma. Since the period of maximal postoperative elevation had been exceeded, it was deemed probable that the further rise from 145 to 214

mg. per cent within 24 hours following injection was related to the parathyroid extract. Return to preinjection levels was delayed beyond 48 hours in three of the five instances. The response of euparathyroid subjects given 200 units and 500 units of parathyroid extract is compared in Fig. 4.

TABLE III. SERUM MUCOPROTEIN LEVEL FOLLOWING MAJOR SURGICAL PROCEDURES

OPERATIVE PROCEDURE	PREOPERATIVE MEAN (MG./100 ML.)	MAXIMUM LEVEL	DAY OF MAXIMUM RESPONSE	RISE (MG./100 ML.)	INCREMENT (% OF INITIAL VALUE)
Parathyroidectomy	116	247	4th	131	113
Parathyroidectomy	106	189	3rd	83	78
Parathyroidectomy	110	151	5th	41	37
Thyroidectomy	100	160	1st	60	60
Thyroidectomy	124	197	3rd	93	75
Thyroidectomy	73	110	6th	37	51
Thyroidectomy	105	214	1st	109	104
Thyroidectomy	104	178	3rd	74	71
Gastrectomy	107	178	3rd	71	66
Gastrectomy	81	214	4th	133	164
Gastrectomy	128	236	4th	108	84
Gastrectomy	48	80	4th	32	67
Gastrectomy	102	197	4th	95	93
Cholecystectomy	103	141	1st	38	37
Cholecystectomy	137	250	2nd	113	83
Cholecystectomy	104	175	3rd	71	68
Common duct exploration	78	167	1st	89	114
Esophageal exploration	88	167	4th	79	90
Sympathectomy	77	160	3rd	83	108
Sympathectomy	77	170	5th	93	121
Sigmoid carcinoma resection	179	302	5th	123	69
Abdominoperineal resection	106	230	2nd	124	117
Abdominal laparotomy	128	203	2nd	75	59
Sigmoid resection	112	143	5th	31	28
Hemicolectomy	189	240	4th	51	27
Leg amputation (gangrene)	137	279	4th	142	104
Hysterectomy	83	139	4th	56	68
Femoral hernia	92	116	4th	24	26

DISCUSSION

The serum mucoprotein fraction determined turbidimetrically was remarkably constant in stable hospitalized patients under fasting basal conditions. In contrast, the considerable increment during the day in approximately 50 per cent of the individuals so studied suggests a significant diurnal variation, which is not influenced by meals. The gradual progressive rise, which was consistently maximal in late afternoon, appears to be a biologic rather than a methodologic phenomenon. Whether this lability is a response to exercise or to other undefined stresses remains unexplained.

Stress has been implicated by many investigators in the production of increased serum mucoprotein concentration in a wide variety of unrelated conditions.[2-4, 7-13] Stress may act either by way of an adrenal influence on serum protein formation or may in itself cause tissue breakdown, with the resultant

products serving as components of the serum mucoproteins. Since such adrenal-mediated mucoprotein response may be independent of a particular adrenocortical steroid secretion, study was directed to adrenal response to ACTH rather than the effect of individual steroids.

An immediate result of adrenal stimulation is the elevation of the mucoprotein level, observed following ACTH infusion. It was clearly related to dosage, requiring two consecutive daily infusions of 30 to 40 units for maximal response. The observation by others[7, 12] of sustained mucoprotein elevation after ACTH treatment of acute rheumatic fever, despite clinical arrest of disease, may be explained by an independent adrenal cortical influence on mucoprotein level. In contrast, the remarkably prompt and transient mucoprotein elevation following injection of parathyroid extract probably results from a direct effect of the hormone on connective tissue, previously demonstrated experimentally by Engel.[13]

The mucoprotein levels following surgical operation showed a biphasic elevation. The rapid early rise is probably mediated by an immediate adrenal cortical mechanism caused by operative stress. It compares with the rise of similar magnitude following ACTH injection and other evidence of increased adrenocortical activity in the hours following surgery.[14] It is probable that the subsequent elevation, of considerably greater magnitude and sustained for several weeks, results from tissue injury and reparative processes.

The mucoprotein elevation following injection of bacterial pyrogen paralleled the febrile response only initially. After multiple injections, mucoprotein elevation persisted or recurred in the absence of fever. This may be explained in part by tissue breakdown in the febrile state and in part by an adrenal cortical mechanism. Adrenal response to injected bacterial pyrogen has been recently demonstrated by increase of circulating adrenal corticosteroids.[15] Moreover, mucoprotein elevation following immunization (and therefore independent of fever) has been observed experimentally in the dog and guinea pig.[3, 11, 16]

Administration of histamine has been used as a stress stimulus, but more recently it has been shown that histamine does not excite adrenal cortical secretion.[15] The response of mucoprotein to histamine infusion was not clearcut.

SUMMARY

The serum mucoprotein content was constant in the fasting basal state in stable hospital patients. Slight elevation was observed in elderly individuals. A diurnal increase, not influenced by meals, was observed in late afternoon. No significant mucoprotein elevation followed histamine injection. Bacterial pyrogen and ACTH elicited mucoprotein increments of 50 per cent. A mucoprotein rise of similar magnitude in the first 24 hours following major surgical procedures was followed by a further rise on the fourth postoperative day, which was sustained. Elevation upon administration of parathyroid extract was prompt but transient. These findings were interpreted to indicate that adrenal stimulation as well as connective tissue breakdown cause elevation of the level of serum mucoprotein.

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A NOTE ON THE DISTRIBUTION OF THE SERUM PROTEIN FRACTIONS IN APPARENTLY NORMAL PERSONS IN CARACAS

JORGE VERA E., M.D., AND MARCEL ROCHE, M.D.
CARACAS, VENEZUELA

IN THE course of an investigation of the serum protein pattern in diverse pathologic states, it was necessary to establish the normal pattern in Venezuela. As the results varied significantly from those reported generally in the literature from various countries,[4-9] we wish to report them here.

MATERIALS AND METHODS

The group studied consisted of men and women chosen among blood bank donors from Caracas. The mixture of races in Venezuela varies with the region involved. In Caracas it is widespread in the general hospital population, so that it is impossible to guess the various proportions of the Indian, white, or Negro blood in a single individual. Of the 80 sera studied by paper electrophoresis, 21 came from subjects considered by the observers to be pure white, 7 from presumably pure Negroes, and 52 from "morenos," with varying admixtures of the three races. None of the subjects studied were habitual donors.

All subjects were in apparent good health and with a blood hemoglobin level above 12 Gm. per 100 ml. These subjects were submitted to a history and physical examination; a nutritional inquest was performed on each.

Nutrition in all subjects studied was adequate, as judged by interview and by clinical appearance. Details of the nutritional and other clinical and laboratory studies will be published later.[1]

In a group of 38 subjects, electrophoretic patterns were carried out by the optical method in an Aminco "portable" electrophoresis apparatus, with barbital-sodium barbital buffer pH 8.6, ionic strength 0.1 μ. In another group of 80 subjects, paper electrophoresis was performed in an Elphor apparatus,* according to the method of Grassmann and Hannig.[2] Total proteins were measured by the biuret method, checked against the micro-Kjeldahl method. The paper strips were stained with amido-black and washed with methanol. Photometri readings were performed in an Elphor photometer. The curves

TABLE I. SERUM PROTEIN LEVELS IN 80 NORMAL BLOOD DONORS IN CARACAS (PAPER ELECTROPHORESIS)

TYPE OF PROTEIN	GM./100 ML.		% OF TOTAL PROTEINS	
	AVERAGE	STANDARD ERROR	AVERAGE	STANDARD ERROR
Albumin	4.02	0.03	55.16	0.38
Alpha 1 globulin	0.27	0.01	3.70	0.11
Alpha 2 globulin	0.55	0.02	7.49	0.18
Beta globulin	0.83	0.01	11.35	0.15
Gamma globulin	1.63	0.04	22.30	0.31
Total proteins	7.30	0.11	—	—

From the Instituto de Investigaciones Médicas, Fundación Luis Roche, Plaza Morelos and Banco de Sangre Municipal, Caracas, Venezuela.

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*Bender und Hobein, München.

of both the optic and the paper methods were measured by planimetry, according to the method of Tiselius and Kabat.[3] The resulting percentages were not multiplied by any coefficient.

RESULTS

In Table I are shown the average results of paper electrophoretic analysis of serum proteins in 80 normal subjects in Caracas.

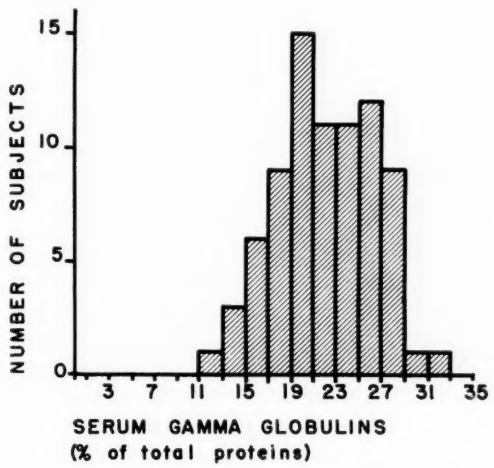

Fig. 1.—Distribution curve of serum gamma globulin concentration in apparently normal Venezuelans.

The histogram in Fig. 1 shows the distribution of the gamma globulin values in this group. In Table II may be seen the results of serum protein analysis by the optical method in a different group of 38 normal subjects.

TABLE II. SERUM PROTEIN LEVELS IN 38 NORMAL ADULTS IN CARACAS (OPTICAL ELECTROPHORESIS)

		GM./100 ML.		% OF TOTAL PROTEINS	
	TYPE OF PROTEIN	AVERAGE	STANDARD ERROR	AVERAGE	STANDARD ERROR
Ascending	Albumin	3.85	0.080	52.14	0.84
	Alpha globulin	0.87	0.028	11.81	0.38
	Beta globulin	1.09	0.033	14.75	0.45
	Gamma globulin	1.58	0.065	21.30	0.68
Descending	Albumin	3.99	0.060	53.96	0.81
	Alpha globulin	0.81	0.029	11.02	0.39
	Beta globulin	1.10	0.031	14.85	0.42
	Gamma globulin	1.49	0.058	20.17	0.69
	Total proteins	7.39	0.032	—	—

DISCUSSION

The total protein concentrations lie within the ranges given by other authors,[4-9] as do the concentrations of globulins α, β, and γ. The average concentrations of the gamma globulins, on the other hand, is above the level usually given as the upper limit of normal by most authors.[4-9]

TABLE III. DISTRIBUTION OF PROTEIN FRACTIONS IN NORMAL INDIVIDUALS FROM VARIOUS COUNTRIES

REFERENCE NUMBER	COUNTRY	METHOD*	NO. OF SUBJECTS	TOTAL PROTEINS (GM./100 ML.)	DISTRIBUTION OF PROTEINS (% OF TOTAL PROTEIN)				
					ALBUMIN	GLOBULINS			
						α_1	α_2	β	γ
4	U.S.A.	O	80	7.23 ± 0.54	56.0 ± 2.8	7.2 ± 1.3	8.8 ± 1.9	13.1 ± 2.5	14.7 ± 2.6
5	U.S.A.	O	13		60.0	3.6	9.1	12.5	14.9
6	Chile	O	25	7.34 ± 0.1	57.6 ± 0.6	7.4 ± 0.4		14.3 ± 0.4	20.7 ± 0.6
7	Belgium	P	13		61.7 ± 3.1	4.90 ± 0.7	8.10 ± 1.4	10.10 ± 1.1	15.20 ± 1.9
8	Spain	O	18		62.0	6.5	4.5	10.5	15.2
9	Spain	P	15	7.38	57.6	4.40	9.16	11.44	17.44

*O = Optical. P = Paper.

While a large number of publications deal with the theoretical aspects of electrophoretic procedures, only a few give a significant number of results performed in normal individuals. Thus, after eliminating the reports dealing with less than 10 subjects and those dealing with plasma analysis, we were left with six references, from various countries, presented in Table III. Additional references with a smaller number of patients are quoted by Reiner and associates.[4] The results in those references generally agree with the values given in Table III.

It may be seen that the highest values for gamma globulins in normal subjects were found by the group in Chile, a country in some respects similar to ours. On the other hand, the small standard deviation shown by these authors indicates that very few of their values for gamma globulin lie above 21.9 per cent.

In the histogram shown in Fig. 1 it is clear that many of our values lie well above this, in a region considered generally as pathologic.

The results obtained in the group studied by optical method are in general similar to these obtained by paper strip analysis; a high gamma globulin value was also found here, with 7 values above 25 per cent and 12 values between 20 and 25 per cent.

What the factors may be which condition the increase in gamma globulins in our material is at present obscure. In Caracas, *Schistosoma mansoni* infestation is frequent; an effort was made to rule it out by questioning closely the patients about baths in potentially polluted waters and eliminating those who gave positive history. Malarial infestation is at present a rarity in Venezuela. Necator infestation is infrequent in subjects from the city, but it is possible that other more frequent infestations, such as with *Ascaris lumbricoides,* may be responsible. The present study cannot determine what role race may play, since the different groups are insufficiently large and poorly defined. It may be interesting, however, that the average gamma globulin value in the group of 21 whites was 20.91 per cent, in the 52 mestizos 22.59 per cent, and in the 7 Negroes 24.09 per cent.

One would rather tend to suspect some quantitative or qualitative difference in dietetic habits as a factor for the finding of hypergammaglobulinemia.

Le Gac and his co-workers in Oubangui[10] have found values for gamma globulins which resemble very much our own in Negroes in apparent good health who "did not present clinically detectable pathologic signs." In their material, geophagism, malaria, multiple parasitism, diarrhea, and dysentery were frequent. The ratio between animal and vegetable proteins was very low. Anderson and Altmann[11] studied 13 Bantu children with malignant malnutrition and 9 with nutritional edema and found gamma globulin values above 20 per cent in the majority, above 30 per cent in 9, with the "normal" value listed as 13.2 per cent, with a range of 8.8 to 16.0 per cent. It is interesting that many of those cases showed a persistent hypergammaglobulinemia after clinical cure.

At any rate, the findings herein reported may serve as a caution to those workers studying changes in the globulin fractions in various disease states in tropical regions similar to ours. Thus, for example, Berning in Venezuela[12] and Trincao and associates, in Portuguese Somaliland[13] reported elevated levels of serum gamma globulins in tropical myocarditis and in hookworm disease, respectively. The gamma globulin levels reported by these authors are well within the range found by us for "normal" individuals in Venezuela.

SUMMARY

The gamma globulin levels in "normal," well-nourished persons in Caracas were found to be generally well above those generally reported in the literature. Care should be taken in the interpretation of abnormally elevated gamma globulin levels in countries similar to Venezuela in climate, nutrition, and general habits.

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CONTRIBUTION TO THE PHYSIOLOGY OF CAPILLARY RESISTANCE IN THE HUMAN

JENÖ KRAMÁR, M.D., V. WILLIAM MEYERS, M.D., AND
MARGARET SIMAY-KRAMÁR, M.D.
OMAHA, NEB.

CAPILLARY resistance has been the subject of numerous studies ever since its measurement became practical. Many physiologic and pathologic factors were considered to influence this phenomenon; yet, without a basic knowledge of its underlying mechanism no uniform comprehension or proper interpretation was feasible. Recent advances indicating that capillary resistance is subject to endocrine influence[1-3] and to characteristic changes upon the application of various forms of stress ("capillary stress response")[4-9] seem to justify an attempt to revise our pertinent knowledge on the human subject from both physiologic and pathologic points of view. The present study resulting from observations of healthy individuals during a period of four years intends to contribute to the elucidation of the *physiologic* aspects of this problem.

METHOD

Capillary resistance was tested with the negative pressure method on the forearm. Two types of apparatus were used. For screening and mass work the electrically-powered device originally constructed for testing experimental animals was used. Individual follow-ups and studies on stress were made by means of portable apparatuses. Both devices consisted of a vacuum reservoir which was connected, respectively, to a hand- or motor-powered suction pump in one direction and to a plastic suction cup with an inner diameter of 7 mm. and an outer diameter of 13 mm., in the other direction. An intercalated mercury manometer indicated the degree of suction applied. The maximum suction yielded by the portable apparatus was -65 cm. Hg, that produced by the motor-powered instrument -70 cm. Hg. The least negative pressure applied for 60 seconds capable of eliciting one or more petechiae within the area of the inner circle of the suction cup was considered the value of capillary resistance. Since this value is subject to great variations depending on the part of the body tested, the standard site of testing was selected with great care. A triangular area was delineated on the proximal volar aspect of the forearm. The base of this triangle runs parallel and approximately 2 cm. distal to the cubital flexion crease, the sides being the creases of the extensor and flexor groups of muscles of the wrist and the hand. The apex of the triangle is then in about the middle of the forearm. It was made a rule to avoid use of the same skin areas within 48 hours following a testing; in serial readings, therefore, the area tested was marked and the adjacent one was used for the next testing.

From the Department of Pediatrics, the Creighton University School of Medicine.

This work represents part of a project supported by the Cardiovascular Section of the Division of Research Grants of the U. S. Public Health Service and by the Creighton University School of Medicine. Parts of this experimental work were carried out, in partial fulfillment of their senior thesis requirements for the degree of Doctor of Medicine at the Creighton University School of Medicine, by the following: L. H. Boggs, F. A. Bertolucci, C. T. Brierty, S. J. Campagna, J. J. Carduff, P. J. Connor, A. W. Davis, J. B. Kilmer, T. W. Klein, N. L. Nelson, G. F. Rutten, H. E. Storino, D. E. Sullivan, D. J. Sullivan, L. F. Thomas, and S. A. Zarbano.

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With the small suction cup used, about 25 individual readings may be obtained in this manner on one arm, enough for about five individual testings. It was essential to avoid the possible error resulting from spasm of the precapillaries in certain (vasolabile) individuals. This phenomenon was described in detail in a previous paper.[6] In order to control the reliability of the method, numerous parallel measurements were made within the area described above; in addition both arms were tested comparatively on 80 subjects to assess this variable.

PROCEDURE

Observations were made on 450 healthy white persons; 276 were medical students, 70 were inmates of Boys Town and 75 those of St. James Orphanage, 9 were student nurses, and the remaining 20 were other healthy volunteers, the authors included. Fifty-one were females and 399 males. The age distribution at the beginning of this study was the following: 3 to 5 years, 15; 6 to 10 years, 37; 11 to 14 years, 36; 15 to 20 years, 65; 21 to 30 years, 286; 31 to 40 years, 6; 41 to 56 years, 5. Two hundred and four persons were tested only occasionally, and 246 were tested at regular intervals ranging from 1 to 3 months during a period of 2 to 4 years. At times of striking changes in capillary resistance, tests were made more frequently. Seventy persons of the latter group were used for experiments with a specific purpose.

1. In five persons the value was measured twice daily (at 7:30 A.M. and 5:30 P.M.), during a period of 22 days (two subjects) and 14 days (three subjects), to demonstrate the possible changes which may parallel the 24-hour rhythm of many other body functions. The subjects observed a strict routine in activity and rest, and great care was taken to avoid any departure from this routine.

2. Ten persons were tested daily for approximately three weeks to study the daily fluctuations which may occur under strictly regulated, standard conditions.

3. Four persons were regularly followed at 10-day intervals during a period of 21 months to ascertain the seasonal variations.

4. On ten persons whose daily routine and environment were well known and controlled, the possible effect of any gross change in their daily life was studied. Four subjects were identical with those serving for the study of seasonal variation. They were tested daily in repeated periods of 2 to 3 weeks during the course of their long-term follow-up. Two other persons tested themselves every third day for a period of ten weeks, including two subperiods of school activity separated by a ten-day recess. Four subjects observed themselves daily during two periods of 40 and 30 days, respectively. The experiments were timed in such a manner that the midpoint of each period coincided, respectively, with the final school or medical board examination.

5. Three individuals, after their base line of capillary resistance had been established, were subjected to intensive muscular exercise (long distance swim), and capillary resistance was tested in daily intervals for at least one week thereafter.

6. Forty-three volunteers (medical students, student nurses, and the authors) served for a more detailed study of the "individual" capillary stress response. The following standard method was worked out. A control base line of capillary resistance was established by daily testing for one week; then the experimental person was subjected to ultraviolet irradiation. A Hanovia Sunnette apparatus was used at a distance of two feet. The chest and the face were exposed for two minutes and the back for three minutes. The site of testing the capillary resistance was carefully protected. A mild erythema with no discomfort was produced. Capillary resistance was again measured in daily intervals for at least one week. The experiment was repeated in five persons after an interval of three weeks and in four others after ten months.

RESULTS

Under strictly physiologic conditions and with use of the above-described procedure of testing, capillary resistance values were found to range between 3 cm. and 46 cm. Hg. The adjective "strictly physiologic" will imply that,

to the best of our knowledge, no exogenous or endogenous factors were present which may represent an *unusual* stress to the given individual, even though this stress may commonly be considered physiologic. Values beyond 50 cm. Hg were found only under pathologic conditions. The distribution of the readings in 450 persons is given in a histogram (Fig. 1). Since for clinical purpose the results of capillary resistance tests are usually classified as being "low," "medium," or "high," it is desirable to express the data of the histogram in these terms also. Considering capillary resistance *low* when it measured 9 cm. or less, *medium* when it ranged from 10 to 25 cm., and *high* when it was 26 cm. or more, the over-all distribution of values is the following: *low*—16.3 per cent, *medium*—72.6 per cent, *high*—11.1 per cent.

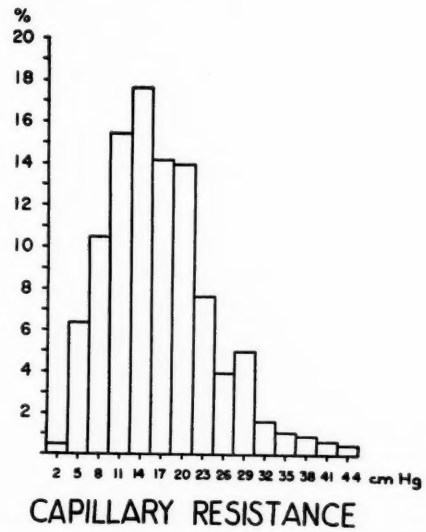

Fig. 1.—Per cent frequency distribution of capillary resistance values obtained on 450 healthy persons. Each rectangle represents the percentage of subjects showing capillary resistance level within a range of 3 cm. Hg (e.g. "2 cm." indicates the range 1-2-3 cm.).

In view of the physiologic fluctuations of capillary resistance in the same individual, it was realized that these figures constitute only a broad generalization. Before further study was undertaken, the reliability of the method and the physiologic variations of capillary resistance had to be analyzed.

Control of the Method.—It was found, first, that when the tests were repeated on the same subject in the delineated area of the same arm, the results may deviate by ±1.0 cm. Hg at most. This may be considered the error of the method. Secondly, when both arms were tested comparatively, an average difference of 1.6 cm. was found, the maximum deviation being 3.5 cm. There was no correlation between the higher figure and the predominate arm; in daily follow-ups, the higher figures were not even found consistently in the same arm.

Diurnal Variations.—As to the physiologic fluctuation of capillary resistance, the diurnal variations were analyzed first. On all five subjects tested, the morning values were found consistently higher than the evening values.

The average difference and the standard deviation (in brackets) were the following: 3.8 cm. (±1.00 cm.); 5.2 (±2.65); 7.0 (±2.45); 5.1 (±1.41); 6.0 (±1.73). Fig. 2 shows one of these observations.

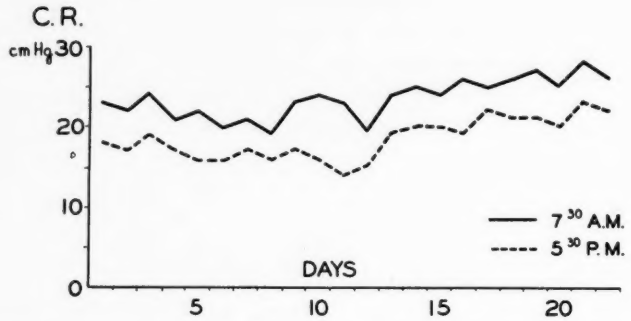

Fig. 2.—Diurnal variations in the capillary resistance of a 39-year-old healthy man. Capillary resistance is consistently higher in the morning.

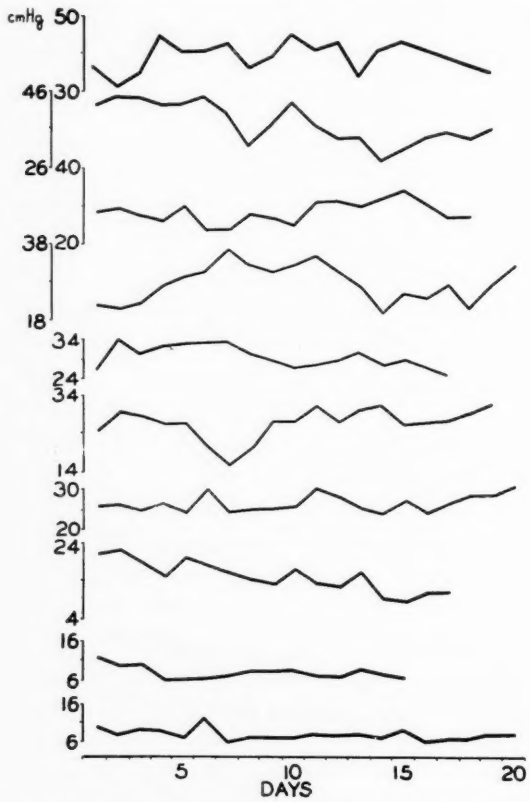

Fig. 3.—Day-to-day variations of capillary resistance in ten healthy residents of Boys Town, Nebraska, of 11 to 16 years of age, living under ordinary routine circumstances. Individual capillary resistance levels are maintained despite physiologic fluctuations.

Day-to-Day Variations Under Constant Conditions.—The day-to-day variations were studied on 10 subjects during a period of 15 to 21 days. As shown

in Fig. 3, these variations have an individual character; in some persons the fluctuations were wide, amounting to 10 or more centimeters, whereas in others they were more shallow, giving rise to a rather smooth curve. Fig. 3 also demonstrates that despite these fluctuations, a certain constant level of capillary resistance is maintained in each individual. The two curves at the bottom of the figure, for example, run a low base line, the third from below a medium one, and the others represent high base lines.

It is possible that fluctuations of the capillary resistance may assume a different character under pathologic conditions, and the question may arise in the future whether or not the fluctuations found in a given group of individuals should be considered within the normal range. For this purpose we added to the above ten persons 38 more subjects whose capillary resistance had been tested at least ten times and calculated the standard deviation as well as the coefficient of variation. The values $\sigma = 1.4$ and $V = 8.7$ may thus serve for a basis of comparison.

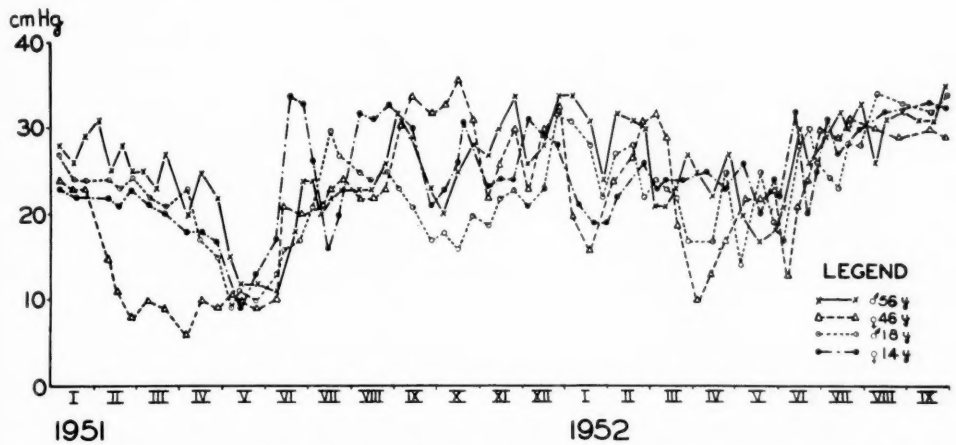

Fig. 4.—Seasonal variations of capillary resistance of four members of a family. Note the tendency of capillary resistance to decrease in late winter and early spring in both years. The return of capillary resistance to former higher levels occurred in June and coincided with the beginning of outdoor activity.

Seasonal Variations.—Fig. 4 summarizes the results of 21 months' observation made by one of the authors on himself and on three members of his family. The readings were taken in approximately ten-day intervals; the fluctuations therefore show a much wider range than with daily testings. Yet even so it demonstrates that capillary resistance tends to be lower in all persons during the months March, April, and May and higher in the rest of the year.

Effect of Gross Change in the Daily Routine of Life.—In short-term observations on eight persons, it was found that as long as the daily routine of the experimental person remained uniform, capillary resistance did not deviate markedly from the base line. A "speeding up" in the daily routine with decrease of the time devoted to relaxation and rest caused the capillary resistance

to increase, a "slowing down" to return to the base line. In times of constant tension and overwork, capillary resistance values approaching pathologic levels (45 to 50 cm. Hg) were repeatedly observed by one of the authors on himself with a return of these high values to his average base line of 27 to 32 cm. after relaxation. This was demonstrated also in the experiments on two medical students during the alternating periods of school activity and vacation. During the first three weeks of December, 1952, and in early January, 1953, these persons' average levels were 32 and 25 cm., respectively, whereas during the 10 days of Christmas recess they were 25 and 19 cm. In Fig. 5 the effect of mental and psychic strain of the medical board examinations is shown in one of the four subjects studied. The marked increase of capillary resistance coinciding with the examinations and the return to the base line thereafter were conspicuous.

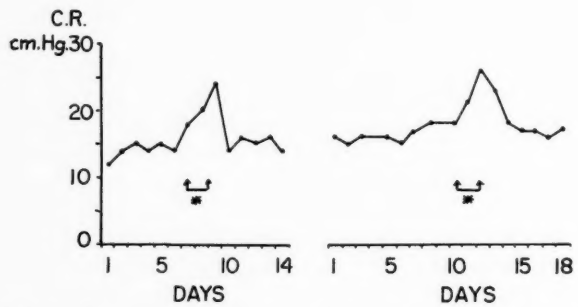

Fig. 5.—Effect of mental and psychic strain of the Medical Board examination (*) on the capillary resistance of a 30-year-old senior medical student. The two parts of the experiment are separated by a four-week interval.

Response to Stress.—

A. Physical exercise: In Fig. 6 the changes in capillary resistance occurring upon intensive physical exercise (long distance swim) are shown on three experimental persons. The capillary resistance rose in all three persons, the increased resistance lasting for 24 to 48 hours. In the experiment on Subject J. K. the increase was followed by a temporary decrease below the base line (biphasic reaction, vide infra). This response was found in February 1954, at a time when, prior to this experiment, the otherwise well-trained subject interrupted his regular training for one month in order to render the physical stress more effective. In January 1955, the same person, this time in a well-trained condition, repeated the experiment, applying the same amount of stress (one mile), with the result that no significant change occurred in his base line.

B. Ultraviolet irradiation: In the evaluation of the results of the experiments on 43 subjects with ultraviolet light as a standard stress, only changes of more than 5 cm. Hg above or below the maximum deviation from the mean of the control base line were considered significant. When all individual experiments were grouped, four patterns resulted (Fig. 7). Ten subjects responded with an increase, seven with a decrease, and 23 did not change.

while in three the response was biphasic, i.e., after an initial rise the value dropped below the base line before returning to it. The degree of rise or decrease varied between 6 to 19 cm. Hg, the mean being 9.5 cm. The time which

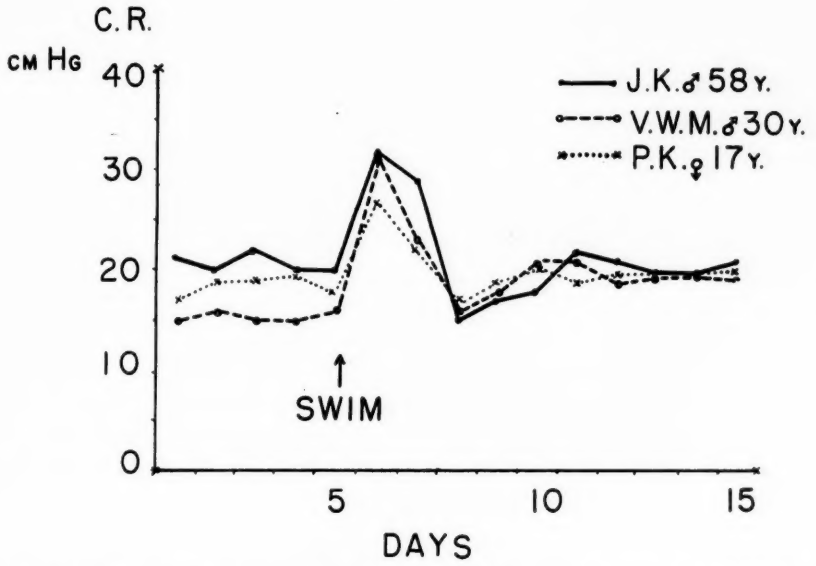

Fig. 6.—Effect of heavy muscular work (one mile swim) on the capillary resistance of three human subjects.

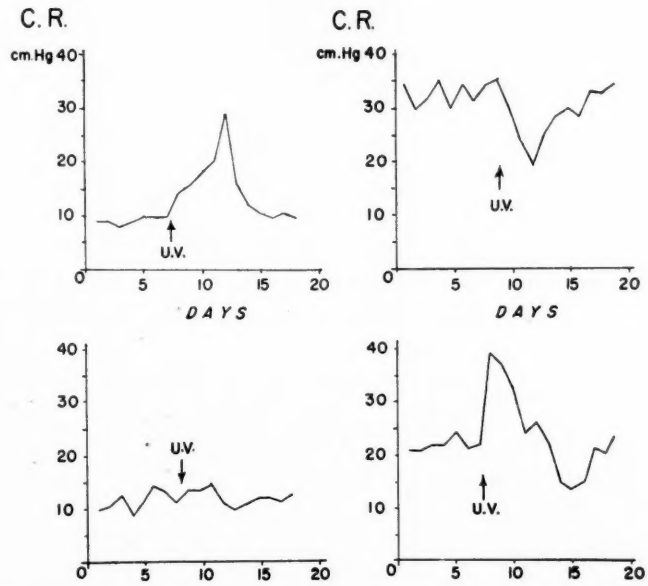

Fig. 7.—Typical patterns of capillary stress response to ultraviolet irradiation in the human being: increase, decrease, no change, biphasic response.

elapsed until the value returned to the base line ranged, as a rule, from two to seven days (mean 4.3 days). One biphasic response lasted exceptionally long (12 days).

When irradiation was repeated, the patterns shown in Table I resulted:

TABLE I

EXPERIMENTAL SUBJECT	PATTERNS OF RESPONSE TO: FIRST IRRADIATION	IRRADIATION REPEATED 3 WEEKS LATER	IRRADIATION REPEATED AFTER 10 MONTHS
1.	Biphasic	Biphasic	—
2.	Increase	Increase	—
3.	Increase	No change	—
4.	Increase	No change	—
5.	Increase	Decrease	—
6.	No change		No change
7.	No change		No change
8.	Increase		Increase
9.	No change		No change

Having familiarized ourselves with the physiologic variations occurring in short and long intervals and having learned the patterns of capillary response to stress, it was easy to evaluate the capillary resistance level of the 246 subjects tested periodically during the past three years. In case of any striking deviation from the subject's "usual" individual level, the situation was scrutinized and capillary resistance was tested repeatedly until its return

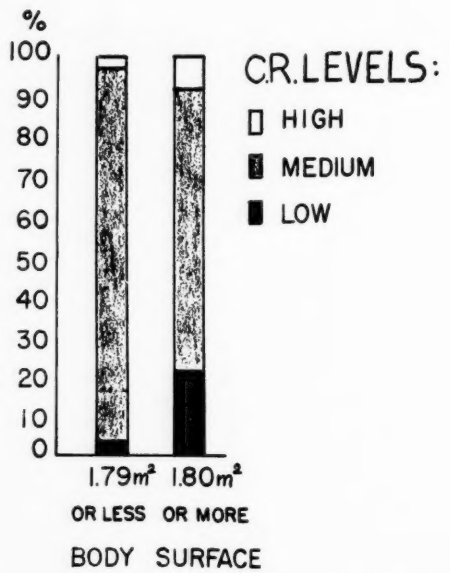

Fig. 8.—Correlation between individual capillary resistance level and body build. Under physiologic conditions, low capillary resistance is most likely to be found in tall and slender individuals.

to the original level. In addition to occasional diseases, unusual physical stress, mental exertion, and psychic trauma were found to cause significant deviations from this level. By recognizing and disregarding these incidental deviations, the individual base line of capillary resistance was found to be *low* in 18.3 per cent of the 246 subjects, *medium* in 67.1 per cent, and *high* in 14.6 per cent.

It should be mentioned that among the 45 persons representing the *low* group there were five whose capillary resistance never rose above 7 cm. Hg (13 tests in each) during 18 months. In two persons the value fluctuated between 3 and 9 cm. (11 tests in each) during one year of observation, with one incidental rise to 20 and 21, respectively, when measurement was made on a day following a strenuous school examination.

With the use of the surface area (DuBois) as an index, an attempt was made to correlate the levels of capillary resistance with the body build. As shown in Fig. 8, the relative frequency of a low base line appears to be higher among subjects with large body surface, i.e., among tall and lean individuals. In order to decide whether or not this difference found in the percentages of low capillary resistance between small and large body surface groups is statistically reliable we applied the formula

$$CR = \frac{(P_1 - P_2) - O}{\sigma_{P_1 - P_2}} \text{ with } \sigma_{P_1 - P_2} = \sqrt{PQ\left[\frac{1}{N_1} + \frac{1}{N_2}\right]} \text{ and found } CR = 2.15,$$

which, with 117 degrees of freedom, indicates that the difference in the percentage is significant.

In 182 persons it was possible to recognize the presence or absence of an allergic tendency. Sixty of them were classified as being "allergic," whereas 122 were labeled as "nonallergic." A comparison of these two groups resulted in the figures shown in Table II.

TABLE II

	LOW	MEDIUM	HIGH
"Allergic" subjects	19.9 per cent	71.8 per cent	8.3 per cent
"Nonallergic" subjects	18.8 per cent	73.0 per cent	8.2 per cent
		ALLERGIC	NONALLERGIC
35 persons with *low* base line		34.2 per cent	65.8 per cent
132 persons with *medium* base line		32.5 per cent	67.5 per cent
15 persons with *high* base line		33.5 per cent	66.5 per cent

DISCUSSION

It is felt that interpretation of these results may be facilitated by a review of the physiology of capillary resistance as it appears in the light of recent advances.

1. *Hormonal Control.*—There is sufficient evidence for hormonal factors influencing capillary resistance. Two hormones seem to play the principal role: cortisone is able to increase and somatotrophic hormone, STH, to decrease capillary resistance.[2, 3, 10] They act slowly but their effect is long lasting. It is postulated at the present time that the actual level of capillary resistance is chiefly the result of a balance between these two hormones.*

2. *Response to Stress.*—Experiments on rats and dogs, and more recently on guinea pigs and rabbits, show that the most varied forms of stress (cold,

*Current studies seem to indicate that for *sudden* and *short* changes in capillary resistance, another set of substances are responsible: vasopressin increases and histamine decreases it.[11, 13] Since the present study deals with the slow and prolonged changes of capillary resistance, this new possibility may be disregarded in this discussion.

muscular exertion, ether anesthesia, anoxia, tissue trauma, emotional exertion, fasting, and realimentation) cause the capillary resistance to change in a definite manner.[4-9, 11-13] This change, termed "capillary stress response," may vary with species, diet, intensity of stress, training, and inherent individual differences; yet, under standard conditions it is rather uniform. The most typical, "full-blown" type of response may be seen in the rat and guinea pig: capillary resistance first increases upon stress and then drops to subnormal levels before returning to the prestress values. Although this is the response of the majority of the animals, occasional individuals, due to innate differences, may be refractory or show only fragments of this average, full-blown response. In the dog, as a rule, one fragment, the increase, is seen most frequently and the full-blown response of the rat is an exceptional one. A study of capillary resistance of 94 patients prior to and following surgical intervention revealed three patterns: increase, decrease, and no change.[7]

It is a well-established fact that corticoids are elaborated in increasing amounts during stress. In certain types of stress (e.g., tissue trauma) impaired conjugation and excretion of these substances may also add to the increase of their concentration in the body.[14] On the other hand, there is indirect evidence for STH also playing a role in stress situations, especially in the recuperative phase.[3, 15]

Analyzing our results in the light of these findings, the *diurnal changes* of capillary resistance should be considered first. They were similar to those described by Roberts, Blair, and Bailey.[16] Capillary resistance was found highest in the morning and decreasing as the day advanced. No explanation has been offered hitherto. In view of our recent knowledge, it seems to be likely that these diurnal changes of capillary resistance are intimately connected with the diurnal cycle in adrenocortical secretion. This latter, postulated by Halberg,[17-18] on the basis of diurnal eosinophil rhythm, as an indirect evidence, was recently proved by Laidlaw and associates[20] with estimation of 17-hydroxycorticoid excretion and by Samuels,[21] Bliss, Sandberg, Nelson, and Eik-Nes,[22] with direct measurement of this substance in the plasma. According to Samuels, the steroid level is low between noon and midnight. Then it begins to rise, obtains a considerable height by 4 A.M., the peak being reached at about 6 A.M., whereupon it gradually decreases to the low noon value. We found the capillary resistance to be high at 7:30 A.M. and low at 5:30 P.M., which is in good harmony with Samuels' data.

The fluctuations of capillary resistance found in *day-to-day observation* may be related to similar fluctuations of adrenocortical function due to the variations in activity and exposure to physiologic strain factors of everyday life. The study of the effect of gross change in the daily routine clearly demonstrated the tendency of capillary resistance to increase with increasing activity. Lack of activity, on the other hand, may result in lower capillary resistance levels.

The observations on the *seasonal variation* (Fig. 4) also seem to bear out this point. Despite the great fluctuations caused by the longer intervals between readings in this experiment, two indentations in the curves are conspicuous,

occurring in March, April, and May in two consecutive years. The first drop in capillary resistance in 1951 was very marked, the second less pronounced. The members of this family, of a rather active type, as a rule, led an unusually monotonous, sedentary life during the winter 1950 and 1951, somewhat less so during the winter 1951 and 1952. The striking surge of capillary resistance and the ensuing higher level in each member of this family was closely connected with the beginning of outdoor activity in early June in both years.

Seasonal variation of capillary resistance has been attributed to lack of the vitamins C and/or P in the diet during the winter season (Schultz,[23] Roberts, Blair, and Bailey[16]). Weld[24] and Roberts, Blair, and Bailey[16] considered the possible role of vitamin D, and Brown and Wasson[25] that of the repeated small respiratory infections occurring in late winter. In our experiment, the vitamin intake was controlled and additional amounts of the vitamins C, P, and D were found ineffective. The 46-year-old female subject, it is true, had a mild rheumatic fibrositis early in 1951, which was accompanied by exceptionally low capillary resistance values. Yet she was healthy in 1952, and the other three members of the family had been so throughout the experiment. The only variables in our experiment were sunshine and physical activity; thus it must be assumed that the seasonal variation of capillary resistance in our four subjects was due to these factors. Both factors, however, are known to increase the activity of the adrenal cortex.[19, 26, 27] The decisive role of physical activity and radiation in the seasonal variation may also be shown by the fact that no such variation has occurred in any members of this family during the last two years since a regular swimming training and several courses of ultraviolet irradiation were carried out in the winter seasons. By this it is not implied that in cases of poor vitamin intake or multiple respiratory infections during the winter season these factors may not play a contributory or even a major role in the seasonal variation. It appears that this phenomenon may be related to a variety of factors depending on the prevailing situation.

It was the seasonal variation which gave us the incentive to study the effects of physical exertion and ultraviolet light more specifically. The stimulating effect of these factors upon the pituitary-adrenal axis was shown by Venning and Browne,[26] Tronchetti and Biancalana,[28] and Flarer and Rabito,[29] and any rise in the capillary resistance found in our stress experiments (pattern 1 in Fig. 7) may be explained on this basis. No explanation can be offered for the other patterns, except for the theoretical assumption that they reflect an individual shift in the corticoid-STH balance.

It should be emphasized that capillary stress response in the human is, despite its many variants, essentially identical with that previously found in experimental animals.[4, 12] The change of patterns observed occasionally after repetition of the stimulus is also in harmony with similar observations in animals.

When during the first screening a number of our experimental subjects were found to show low capillary resistance, this was thought, in accordance with the literature, to be the result of the combined effect of nutritional and seasonal factors. A survey on the diet of our volunteers indicated that the

initial low readings in some subjects were incidental and the subjects really belonged to the medium category. At last a group remained with consistently low capillary resistance, which stayed low irrespective of season, diet, and supplements of vitamins C, P, and D. Pathologic conditions were ruled out by careful physical examination and laboratory tests. Routine ACTH-eosinophil tests (Thorn) resulted in normal adrenocortical response. Since allergy was reported to cause low capillary resistance and since it was also our experience that capillary resistance of children showing *clinical* symptoms of allergy (especially asthma and eczema) is usually low, an attempt was made to correlate capillary resistance level to allergy. Our subjects were healthy, and even though some of them might have had occasional allergic symptoms, in general they had been free from them. The distinction between allergic and nonallergic groups, therefore, had to be made only on the basis of the history. In this survey the incidence of low capillary resistance was found to be the same in both allergic and nonallergic groups. Thus we were left with the ultimate conclusion that 45 of 246 persons have *low capillary resistance as a permanent individual trait.* In an attempt to find a common feature for this group, other than the low capillary resistance, the skin, its thickness and texture, were considered first. So far no evidence was found suggesting the skin as being the common factor and the primary cause of the low capillary resistance. A definite correlation was found, however, with the body build, i.e., constitutional type of these individuals.

The fact that the level of capillary resistance in the human has an individual trait, in other words, that there are people with high and others with medium or low capillary resistance levels apparently has not been known heretofore, although individuals showing "abnormally low" capillary resistance uninfluenced by vitamin supplement have repeatedly been found in former studies dealing with the possible effects of vitamins upon capillary resistance. It is likely that these cases, in fact, represented examples of constitutionally low capillary resistance. It is the task of a current study to find out the underlying cause of these various individual capillary resistance levels, specifically whether or not they are manifestations of a constitutional difference in the degree of adrenocortical activity or in the balance between cortisone and STH.

Our findings have several practical implications. Clinical testing of capillary resistance hitherto served the sole purpose to decide whether or not a bleeding tendency is due to a vascular defect: the capillaries were tested for increased fragility (decreased resistance). The eventuality that *increased* capillary resistance may also indicate a pathologic situation did not even emerge. Present study calls attention to this new diagnostic aspect of the capillary resistance test. On the other hand, it also indicates that low capillary resistance is not necessarily "abnormal." Furthermore, it bears out that evaluation of the individual capillary resistance *level* is a *must* prior to any experiment in which capillary resistance is studied with relation to vitamins, hormones, drugs, or any other influencing factor. By the same token, the

knowledge and recognition of the physiologic variations and the capillary stress response is essential in similar studies. Failure to avoid or recognize any interfering stress effect may jeopardize the results or give rise to misinterpretations.

SUMMARY

This study on 450 healthy subjects during a period of four years is warranted by recent advances in the physiology of capillary resistance which offer new viewpoints for the understanding and interpretation of this phenomenon.

1. Diurnal variations (decrease of capillary resistance as the day advances) seem to be connected with the diurnal rhythm of adrenocortical activity.

2. The daily fluctuations of capillary resistance have an individual trait: some persons show wide fluctuations, others minor ones. They seem to be related to similar fluctuations of adrenocortical activity due to variations in the strain factor of everyday life.

3. To the known possible causes of seasonal variation (dietary deficiency, infections) two factors may be added: physical activity and sunshine. In the described experiments, these two factors were found to play the predominant, if not the exclusive, role.

4. There are people with high and others with medium or low capillary resistance, which is characteristic of the individual, and the fluctuations occur about this average individual level. Particular attention was given to the surprisingly large group of individuals (18.3 per cent) with low levels of capillary resistance. Insamuch as local differences in skin texture as well as allergic and other pathologic conditions were ruled out as possible causes, and since a correlation with the body build was found, a constitutional factor is postulated.

5. Any gross change in the routine of life may cause the capillary resistance to deviate temporarily from the individual level.

6. The human capillary stress response to various forms of stress may show four patterns: increase of capillary resistance, decrease, biphasic reaction, and no change. Essentially the human response is identical with that found in various animal species. It is postulated, for the time being as a working hypothesis, that these patterns of stress response reflect individual differences in the balance mechanism of the two hormones involved, corticoids and the somatotropic hormone.

7. A harmless and feasible form of stress was found in ultraviolet light, which, when applied under standard conditions, is very suitable for studying the human capillary stress response.

8. Knowledge of the individual capillary resistance level, the physiologic fluctuations, and the capillary stress response is essential for any study dealing with problems connected with capillary resistance.

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METABOLIC ALTERATIONS IN SURGICAL PATIENTS

VIII. Studies Involving Iron and Magnesium Metabolism in Patients With Gastrointestinal Drainage

Stanley Levey, Ph.D., William E. Abbott, M.D., Harvey Krieger, M.D., and John H. Davis, M.D.
Cleveland, Ohio

INORGANIC elements have been found to play an important role in the general metabolism of both plants and animals. Iron, in addition to being the prosthetic group for many of the enzymes involved in respiration, is present in hemoglobin. In animals, magnesium is an important ion in the maintenance of normal tissue irritability. It is also essential for certain steps in the metabolism of carbohydrate and constitutes an important fraction of the mineral content of bone. While there have been numerous investigations[1-5] as to the importance of iron and magnesium in the nutrition of both man and experimental animals, there have been few reports concerning the metabolism of these substances in the surgical patient.[6] Because of this, it was decided to study, by means of metabolic balance techniques, the alterations of iron and magnesium metabolism in pre- and postoperative patients.*

METHODS

The four surgical patients used in this presentation were being maintained on balance studies for the investigation of changes in sodium, potassium, chloride, and nitrogen metabolism.† Three of the patients had pyloric obstruction requiring gastric suction and, subsequently, operative intervention. The fourth had intestinal ileus which responded to intestinal intubation without the need of an operation. Three of these individuals were chosen because of the large amount of gastrointestinal fluid which was being removed and the other patient was studied to compare the balances of these ions in a person who had little gastrointestinal tract drainage.

The patients were maintained on the Metabolic Division of the University Hospitals of Cleveland, where accurate measurements of intake and output were obtained. Urine samples were collected daily in two-quart wide-mouth glass jars which had been previously washed well and rinsed thoroughly with double distilled water. Each study day began at 6:30 A.M. and ended the next morning at 6:30 A.M. At the beginning of the study period a distilled

From the Department of Surgery, Western Reserve University, School of Medicine and the University Hospitals of Cleveland, Cleveland, Ohio.

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*In a preliminary note, data on the gastrointestinal losses of copper, zinc, and iron were reported (J. Lab. & Clin. Med. 44: 76, 1954). Later it was discovered that the suction apparatus we were employing had a metal connector between the rubber tubes and that both copper and zinc were being leeched from this brass tube by gastrointestinal drainage. Thus, the gastrointestinal content of both copper and zinc was erroneously high. Neither iron nor magnesium was being removed from the metal connector. Studies are now in progress where the copper and zinc contents of gastrointestinal fluid are being evaluated with use of a suction machine in which all metal connectors are replaced by glass.

†Detailed clinical summaries of three of these patients have been reported earlier along with water and electrolyte balance studies.[8]

TABLE I. THE INTAKE AND EXCRETION OF MAGNESIUM AND IRON IN FOUR SURGICAL PATIENTS

PATIENT	DAY	INTAKE MG.	OUTPUT					VOL. GASTRIC FLUID
			URINE	GASTRIC	FECES*	TOTAL	BALANCE	
			Iron					
H. L., 43-year-old man. Hemigastrectomy and vagotomy, peptic ulcer with pyloric obstruction	− 7	7.35	1.70	4.60	0.4	6.7	+0.65	4,500
	− 6	5.15	1.80	5.80	0.4	8.0	+0.65	2,146
	− 5	5.15	1.30	1.90	0.4	3.6	−2.85	1,970
	− 4	5.15	1.30	9.40	0.4	11.1	−5.95	2,320
	− 3	5.15	1.30	4.30	0.5	6.1	−0.95	1,270
	− 2	7.35	1.00	2.70	0.5	4.2	+3.15	1,650
	− 1	7.35	1.80	1.90	0.5	4.2	+3.15	1,475
	OR	2.10	0.23	0.32	0.5	1.05	+1.05	115
	+ 1	2.10	0.88	0.45	0.1	1.43	+0.67	100
	+ 2	2.30	1.33	0.67	0.1	2.10	+0.20	185
	+ 3	2.30	1.50	0.76	0.1	2.36	−0.06	485
	+ 4	2.75	1.50	0.63	0.1	2.23	+0.52	445
			Magnesium					
	− 7	105.2	90.0	72.0	19.7	181.7	−76.5	
	− 6	74.2	32.0	50.0	19.7	101.7	−27.5	
	− 5	74.2	34.0	19.0	19.7	72.7	+1.5	
	− 4	74.2	57.0	33.0	19.7	109.7	−35.5	
	− 3	74.2	68.0	19.0	6.4	93.4	−19.2	
	− 2	105.2	69.0	22.0	6.4	97.4	+7.8	
	− 1	105.2	36.0	16.0	6.4	58.4	+46.8	
	OR	9.6	8.8	1.5	6.4	16.7	−7.1	
	+ 1	9.6	24.0	11.0	0.3	35.3	−25.7	
	+ 2	19.4	8.2	14.0	0.3	22.5	−3.1	
	+ 3	19.4	135.0	12.0	0.3	147.3	−127.9	
	+ 4	33.4	112.0	13.0	0.3	125.3	−91.9	
			Iron					
S. M., 55-year-old man. Intestinal ileus, no operation	1	6.2	0.90	1.50	0.4	2.80	+3.40	930
	2	7.1	1.20	0.70	0.4	2.30	+4.80	420
	3	7.1	0.30	1.40	0.4	2.10	+5.00	1,120
	4	7.1	1.50	0.80	0.4	2.70	+4.40	670
	5	7.1	1.50	0.80	0.4	2.70	+4.40	690
	6	6.4	1.00	0.40	0.4	1.80	+4.60	254
			Magnesium					
	1	88	75.0	7.5	5.9	88.4	−0.4	
	2	101	20.0	5.0	5.9	30.9	+70.1	
	3	101	29.0	24.0	5.9	58.9	+42.1	
	4	101	69.0	16.0	5.9	90.9	+10.1	
	5	101	53.0	14.0	5.9	72.9	+28.1	
	6	94.4	21.0	5.8	5.9	32.7	+61.7	

*Data for feces are the results from two- to six-day pooled samples. See the text for details.

water enema was given and the material discarded. All subsequent stools were collected in wide-mouth glass jars which had been treated as described earlier for the urine containers. The feces were diluted with double distilled water, homogenized, and the total volume was measured. An aliquot was stored in specially cleaned bottles in a refrigerator. At the end of the study period a distilled water enema was given and the excreta treated as described for stool. Aliquots of all stool samples in a given period (two to four days per period, according to the plan of the study) were pooled and a sample of this pool was used for analyses. The total amount of iron or magnesium in the pooled sample was divided by the number of days in the period and an equal portion of the total quantity was assigned to each day of the period.

Analyses of all intravenous fluids, gastric drainage, and feces were made, with use of a Bausch and Lomb medium quartz spectrograph. Samples were arced on rotating plat-

TABLE I—CONT'D

PATIENT	DAY	INTAKE MG.	OUTPUT					
			URINE	GASTRIC	FECES*	TOTAL	BALANCE	VOL. GASTRIC FLUID
			Iron					
F. U., 49-year-old man. Peptic ulcer, temporary pyloric obstruction	−11	6.65	0.91	2.40	0.4	3.71	+2.94	600
	−10	5.15	0.76	3.90	0.4	5.06	+0.09	1,880
	− 9	5.15	0.47	2.10	0.4	2.97	+2.18	1,190
	− 8	4.70	0.26	5.90	0.4	6.56	−1.86	1,475
	− 7	5.15	0.75	3.30	0.4	4.45	+0.70	1,625
	OR	2.1	0.18		0.1	0.28	+1.82	
	+ 1	2.1	0.37	1.90	0.1	2.37	−0.27	120
	+ 2	2.1	0.20	1.40	0.1	1.70	+0.40	270
	+ 3	2.1	0.30	1.00	0.1	1.40	+0.70	240
			Magnesium					
	−11	100.8	99.0	6.0	4.2	109.2	−8.4	
	−10	74.2	91.0	17.0	4.2	112.2	−38.0	
	− 9	74.2	87.0	11.0	4.2	102.2	−28.0	
	− 8	60.2	52.0	15.0	4.2	71.2	−11.0	
	− 7	74.2	0.80	20.0	4.2	104.2	−30.0	
	OR	9.6	34.0		0.1	34.1	−24.5	
	+ 1	9.6	46.0	3.4	0.1	49.5	−39.9	
	+ 2	9.6	56.0	1.4	0.1	57.5	−47.9	
	+ 3	9.6	51.0	1.0	0.1	52.1	−42.5	
			Iron					
E. E., 50-year-old man. Carcinoma of stomach	− 2	6.93	0.81		0.25	1.06	+5.87	
	− 1	7.77	0.19	0.37	0.25	0.81	+6.96	270
	OR	7.37	1.00	3.30	0.01	4.31	+3.06	36
	+ 1	7.35	0.90	1.80	0.01	2.71	+4.64	66
	+ 2	7.35	0.27		0.01	0.28	+7.07	
	+ 3	7.35	1.10		0.01	1.11	+6.24	
			Magnesium					
	− 2	103.2	39.0		2.2	41.2	+62.0	
	− 1	107.1	46.0	4.7	2.2	52.9	+54.2	
	OR	105.2	35.0	1.2	0.1	36.3	+68.9	
	+ 1	105.2	55.0	1.7	0.1	56.8	+48.4	
	+ 2	105.2	41.0		0.1	41.1	+64.1	
	+ 3	105.2	62.0		0.1	62.1	+43.1	

*For footnote see page 438.

form electrodes with use of a Uni-arc exciter.* Aliquots of feces were first dry-ashed in a muffle furnace at 600° C. before analyses. The sensitivity of the method for iron was 0.03 μg per milliliter of solution and for magnesium 5 μg per milliliter of sample. To determine whether there was any leeching of iron or magnesium from the glass connectors or rubber tubing in the suction apparatus used for obtaining gastric drainage, 2 liters of double distilled water, containing enough concentrated hydrochloric acid to bring the pH to about 1.5, was slowly run through the pump for three hours. By analyses, no iron or magnesium was found to be extracted from the suction apparatus.

Since we were primarily interested in determining total balance studies, no attempt was made to separate the urine or gastric drainage into soluble and particulate matter. While none of the samples were noticeably stained with blood, there were probably some blood cells and eroded gastrointestinal lining cells in the samples analyzed.

RESULTS

The balance data from the four patients are summarized in Table I. With the exception of four days for H. L. and two days for F. U., all the patients

*All analyses were done by the National Spectrographic Laboratory, under the supervision of Messrs. W. E. Alsope, J. D. Golland, L. E. Zeeb, and J. T. Rosza.

were in positive iron balance during the entire study. The operative procedure per se did not appear to have any effect on the iron balance. In two of the patients (H. L. and F. U.), during the first few postoperative days, there was a decrease in both the volume of gastric drainage and the amount of iron lost by this route. Usually there was more iron lost by way of the gastric drainage than by way of the urine (from 1.5 to about 5 times as much). In fact, the loss of iron by way of gastric drainage was greater than the sum of the urinary and fecal loss for the first eight days of the study of H. L. (up to and including the day of operation) and during the entire study for F. U. and E. E. In S. M., drainage was obtained as a long intestinal tube passed down the gastrointestinal tract. During the first day it was in the stomach and by the last few

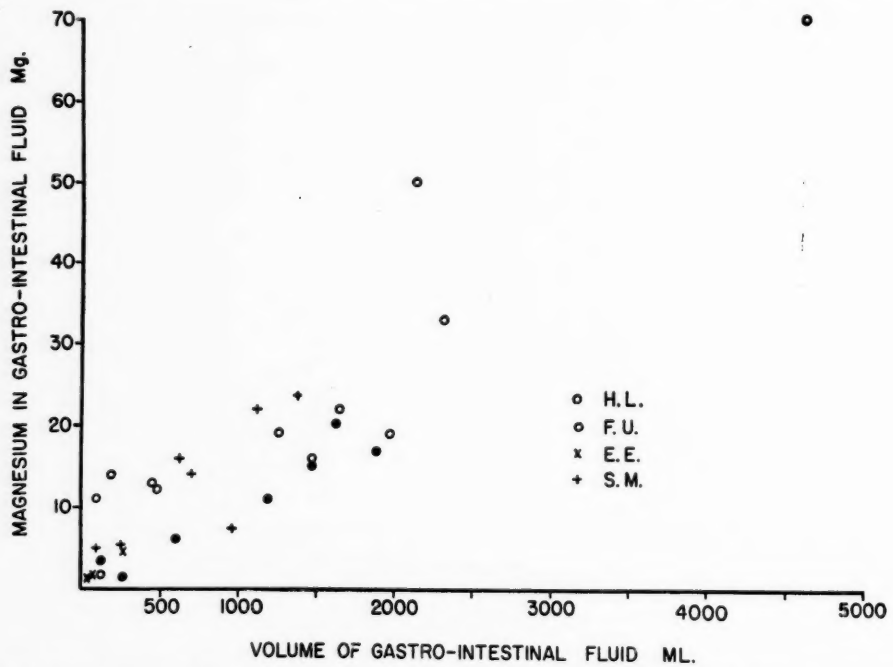

Fig. 1.—Correlation between the volume of gastrointestinal fluid removed and the amount of magnesium lost in this fluid.

days of the study it had reached the terminal ileum. In four of the six study days for this patient, the amount of iron lost in the urine exceeded that in the gastrointestinal drainage.

Two of the patients operated upon, F. U. and H. L., were in negative magnesium balance during all or most of the study. The other two patients, E. E. (operated upon) and S. M. (not operated upon), both showed positive magnesium balance during the entire study. It should be noted that the latter two individuals were receiving about 100 to 105 mg. of magnesium per day in the various intravenous fluids employed. On the other hand, F. U. and H. L. usually received considerably less magnesium in the combination of intravenous fluid given. When the magnesium intake was increased to 105 mg. on the first

two days before operation, in the case of H. L., he showed magnesium storage. The results of this small series might be interpreted as indicating that a surgical patient on drainage needs at least 100 mg. of magnesium per day, if given intravenously, to remain in balance.

A good correlation was noted in the individual patients between the amount of magnesium lost in the gastric fluid and the volume of fluid removed (Fig. 1). While the correlation was good in the case of individual patients, one did not obtain as good a correlation coefficient if all four patients were considered as a single population group. In a previous publication,[8] the losses of sodium, potassium, and chloride were reported for three of these patients (data on E. E. not given), and no correlation was noted between the iron or magnesium balances and those of sodium, potassium, or chloride.

DISCUSSION

According to current concepts, the absorption of iron is regulated by the gastrointestinal mucosa. This area limits the absorption of iron, acting perhaps as a protective mechanism, since the body conserves iron well and has only a limited capacity to excrete it. In fact, it has been suggested that except for bleeding the body loses only small amounts of iron.[9] In the present study the subjects received only the amount of iron which was in the intravenous solutions used. No supplementary iron or magnesium was given. But since this amount was about one-third to one-half of the daily recommended iron allowance for adult men, and since it was administered intravenously, eliminating all possibility of incomplete absorption, two of the four patients showed positive iron balances during the entire study and the other two showed positive balance during most of the study. There was no correlation noted between the amount of iron lost in the gastrointestinal drainage and the volume of fluid removed. The amount of iron found in the feces is of the same general magnitude as that reported by Dubach, Moore, and Callender,[9] who used radioactive iron as a tool. The amounts of iron lost by the surgical patients in the urine, feces, and gastrointestinal drainage are small, but from the nutritional viewpoint, compared to that amount which a normal individual absorbs from his diet, these losses are significant. It should be noted that the iron was determined on whole urine and gastric drainage in which no attempt was made to separate particulate matter (desquamated cells, tissue debris, etc.) whose iron content might be relatively high.

It appears in these studies that magnesium balance is not influenced by the operative stress or disease of the patient but rather is dependent on the intake of this metal. Such impressions are only tentative, since the amount of magnesium that the patients received varied according to the program of intravenous alimentation employed. Patients H. L. and F. U., who had the largest losses of gastric fluid, also showed cumulative magnesium deficits (H. L., 358 mg., and F. U., 270 mg.), But it should also be noted that these patients received reduced amounts of magnesium during their postoperative period, 9.6 mg. per day for F. U. and from 9.6 to 33 mg. per day in the case of H. L. On the other hand, the remaining two patients, S. M., and E. E., showed cumulative

positive balances of 212 and 340 mg., respectively. S. M. had fairly large losses of gastrointestinal fluid and E. E. very little, but the latter patient had a daily magnesium intake of about 105 mg., and S. M. received about 100 mg. per day during most of the study. Thus, it appears that a patient must receive, intravenously, about 100 mg. of magnesium per day to remain in positive balance. This value is considerably less than that reported as the oral magnesium requirement. Tibbetts and Aub[10] found an adult needs about 200 to 300 mg. of magnesium, orally administered, per day. The intravenous requirements probably are less than the oral, since all the material presented is absorbed.

Haynes, Crawford, and DeBakey[6] have reported studies on the urinary magnesium excretion of patients undergoing various surgical procedures and also the excretion of magnesium by patients given 60 to 150 mg. of ACTH per day, for three days, as a substitute for operative stress. The injection of ACTH produced greater metabolic changes in relation to magnesium than did the operations. The patients usually showed decreased urinary excretion of magnesium following the injection of this hormone and the magnesium content of the urine increased for a day or so after cessation of the treatment before returning to its normal level. These investigators suggested that the urinary excretion of magnesium is similar to that of sodium, being under the control of the hormones of the adrenal cortex. In our series, only Patient H. L. had a decreased urinary excretion of magnesium after the operation, but during this time he had developed a fairly significant deficit and also had a marked reduction in the magnesium intake. More studies will have to be done before this point can be defined more accurately.

SUMMARY

Metabolic balance studies concerning iron and magnesium were carried out in four surgical patients with obstruction of the gastrointestinal tract, who were maintained solely on intravenous alimentation.

The patients were in positive iron balance during most of the study. No change in iron balance which could be related to the operative procedure was noted. While the amount of iron lost was not dependent on the volume of the gastrointestinal drainage, this route accounted for the largest losses in most of the patients.

There was a close relationship between the amount of magnesium lost in the gastric drainage and the volume of fluid removed. Whether a patient was in positive or negative magnesium balance did not seem to depend on the operation or volume of gastric drainage but rather on the amount of magnesium he was receiving. Those who received about 100 mg. or more per day in their intravenous fluid remained in positive balance.

We wish to thank Dr. Arthur S. Littell, of the Department of Preventive Medicine, Western Reserve University, School of Medicine, for aid in the determination of the statistical correlations used in this paper.

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EXPERIMENTAL HYDRALAZINE DISEASE AND ITS SIMILARITY TO DISSEMINATED LUPUS ERYTHEMATOSUS

PHILLIP COMENS, M.D.
ST. LOUIS, MO.

INTRODUCTION

REPORTS indicate that 8 to 15 per cent of hypertensive patients treated for several months with large doses of hydralazine hydrochloride (Apresoline) as a hypotensive agent develop a syndrome simulating "collagen disease" and manifested by several of the following symptoms and findings: arthritis, fever, weakness, weight loss, hepatomegaly, splenomegaly, anemia, leukopenia, reversed serum albumin-globulin ratio and, in a few cases, demonstration of the lupus erythematosus cell in the peripheral blood.[1-9] Blood changes include a 4+ cephalin-cholesterol flocculation and increased thymol turbidity of the serum. The clinical similarity of this syndrome to disseminated lupus erythematosus led to attempts to reproduce it in dogs. By the feeding of this drug a syndrome has been induced in dogs, which resembles the human disease, in that the L-E cell appeared through the use of special techniques, and distinctive pathologic changes were found in the kidneys. Because some of the actions of the drug are known, an experimental study of this type of disease is possible.

METHODS

Powdered hydralazine hydrochloride* (Apresoline) in horse meat was fed orally to eight normal dogs; the daily dosage was 200 mg., approximating the maximum human therapeutic dose.[10, 11] Diets consisted of Purina Chow and horse meat. Each animal was kept in a separate cage. No other drugs were administered. Routine studies done in the Barnes Hospital clinical laboratory on the first three animals consisted of complete blood counts, serum cephalin-cholesterol flocculation and thymol turbidity, and plasma cholesterol levels and plasma proteins. Since the findings in the original group of three animals indicated only occasional changes in the laboratory findings, it was not felt necessary to obtain these on the succeeding five dogs. Tissue sections of four kidney biopsies and three post-mortem examinations using hematoxylin-eosin and periodic-acid Schiff stains were examined by members of the Department of Pathology. Normal dogs were sacrificed and tissue sections taken at 2-hour intervals for 24 hours at warm room temperatures as controls for observing progressive post-mortem changes. A total of 24 analyses of plasma proteins was done by paper electrophoresis, with the utilization of normal dog and normal human blood as controls.

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*Supplied by Ciba Pharmaceutical Products, Inc.

L-E cell preparations made twice weekly by the method of Zimmer and Hargraves[12] upon the peripheral blood of each animal totaled well over 200. Standard methods as well as deviations were used in the L-E preparation, i.e., variations of time and temperature of incubation, with use of clotted and unclotted blood and crossing of questionably positive sera with normal canine leukocytes. Variations in methods and techniques, however, failed to show the typical L-E cell.

Following publication of the work of Carrera and associates,[13] the first positive L-E cell preparation was obtained with the utilization of a reversal of their method. The noxious agent responsible for the L-E cell is known to be contained in the serum fraction of the blood, in that serum of a patient with disseminated lupus erythematosus incubated with normal leukocytes will produce L-E cells. Carrera took positive human lupus serum and produced L-E cells from leukocytes of various animals, including the dog. For the present study, blood of dogs receiving hydralazine was drawn and centrifuged. Part of the serum was used fresh and the remainder quick-frozen until needed again. Human heparinized blood was centrifuged in two Wintrobe tubes at 2,000 r.p.m. for 10 minutes and the buffy coat drawn off with a Wintrobe pipette. These normal leukocytes were then mixed and incubated with 1 ml. of canine serum for two hours at 37° C. Following incubation, the specimens were recentrifuged in Wintrobe tubes and duplicate smears made of each specimen. Slides were prepared in the usual manner using Wright's stain. Each preparation was examined for 10 to 60 minutes. Serum of normal dogs incubated with the same human leukocytes served as controls. A standard L-E preparation (method of Zimmer and Hargraves) was made on the blood of each human donor to rule out undiagnosed lupus. Preparations were evaluated by the author and hospital laboratory technicians.

RESULTS

For the first month, the dogs apeared healthy and exhibited normal activity and food intake, and each was quite friendly. The hydralazine camouflaged by horse meat was immediately and eagerly ingested. After approximately 30 days, the animals began to exhibit a decrease in appetite and weight. They refused the horse meat, even without the telltale odor of hydralazine, so that it became necessary to administer the drug in capsular form. Weakness and listlessness became apparent. The animals lay in their pens, rarely rising to all fours. Mucous membranes of the mouth were noticed to be dry and each animal had difficulty in swallowing food or capsules. The dogs became difficult to handle. Reduction of medication frequently alleviated this temperamental state.

In the first animal the daily dose of hydralazine was doubled to 400 mg. after six weeks, and two days later he was found having intermittent convulsions resembling a generalized epileptic seizure. He lay on his side exhibiting clonic movements of all extremities so that he appeared to be running. About the mouth frothy saliva produced a whiskered effect while the jaws snapped open and shut at a rapid rate. The drug was reduced to the original dose, but two days later, he died during another convulsive seizure. Another animal, not included in the table, fed 200 mg. a day for approximately three weeks, was found dead one morning, evidently having experienced the same type of attack, his mouth and chin being covered by a frothy salival beard. These seizures preceded death in all animals which expired. The second dog, followed for four months, showed a weight loss from 37 to 24 pounds; he stopped eating, lay about his pen, and refused to get to his feet even when

prompted. His black coat was noted to have become gray. His general appearance was one of severe illness. Hydralazine administration was stopped

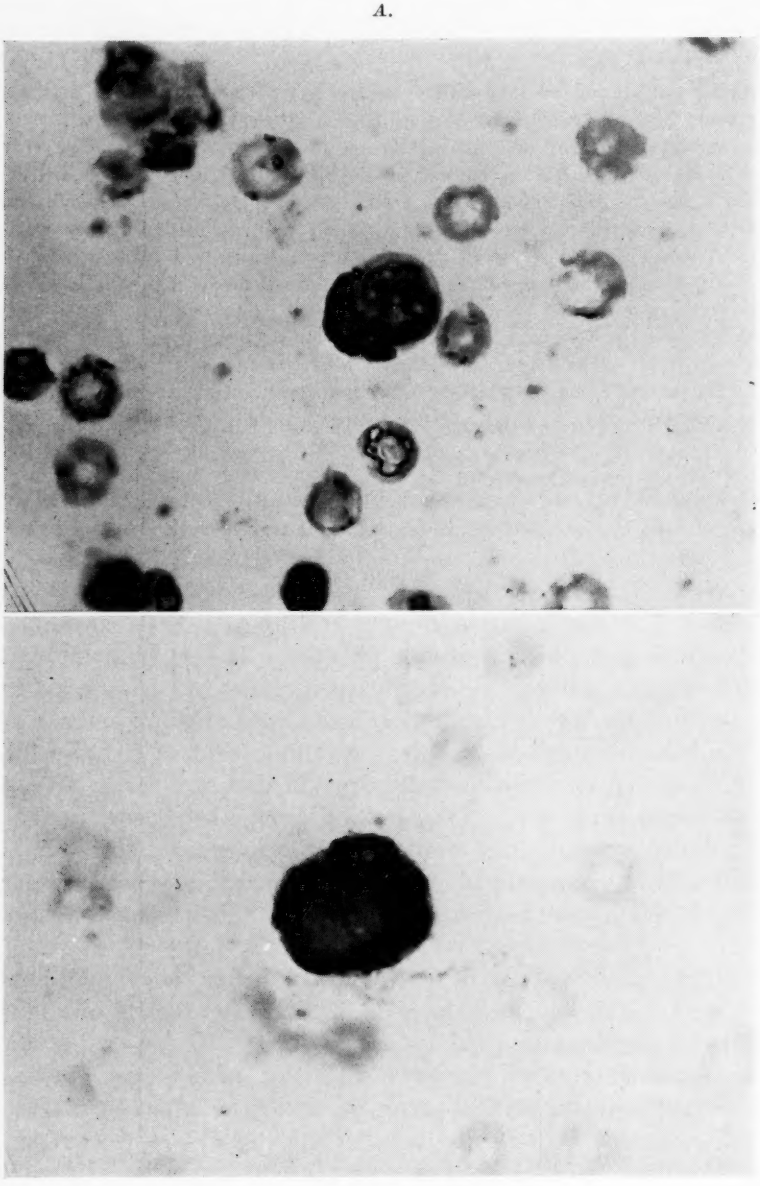
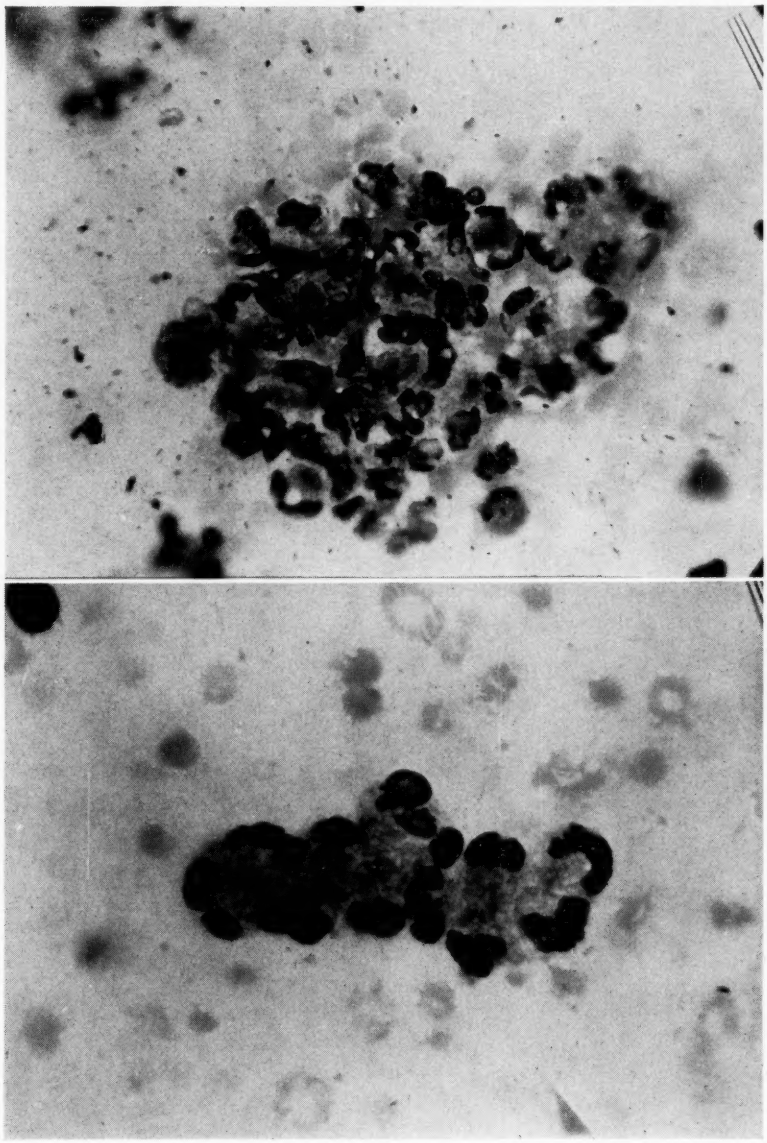


Fig. 1.—L-E preparations of dog serum and human leukocytes after feeding hydralazine for 3 and 4 months. *A*, Single L-E cell from Dog 2 showing typical inclusion body (×1,200). *B*, Same from Dog 3 (×1,350). *C*, L-E cell cluster from Dog 2 (×570). *D*, A cluster from Dog 3 (×1,080).

and the dog made a remarkable recovery. After three weeks, the drug was restarted at 200 mg. with repetition of the above symptoms and signs. He

expired two months later apparently following a seizure. A third dog fed hydralazine for four months showed the same signs after one month; symptoms

C.



D.

Fig. 1, *C* and *D*. (*For legend see opposite page.*)

reappeared in the fifth month when the drug was restarted after discontinuation of the drugs for one month. Dogs 4, 5, 6, 7, and 8 all followed the same general picture, becoming very ill after the first month. Dog 4 is the only one

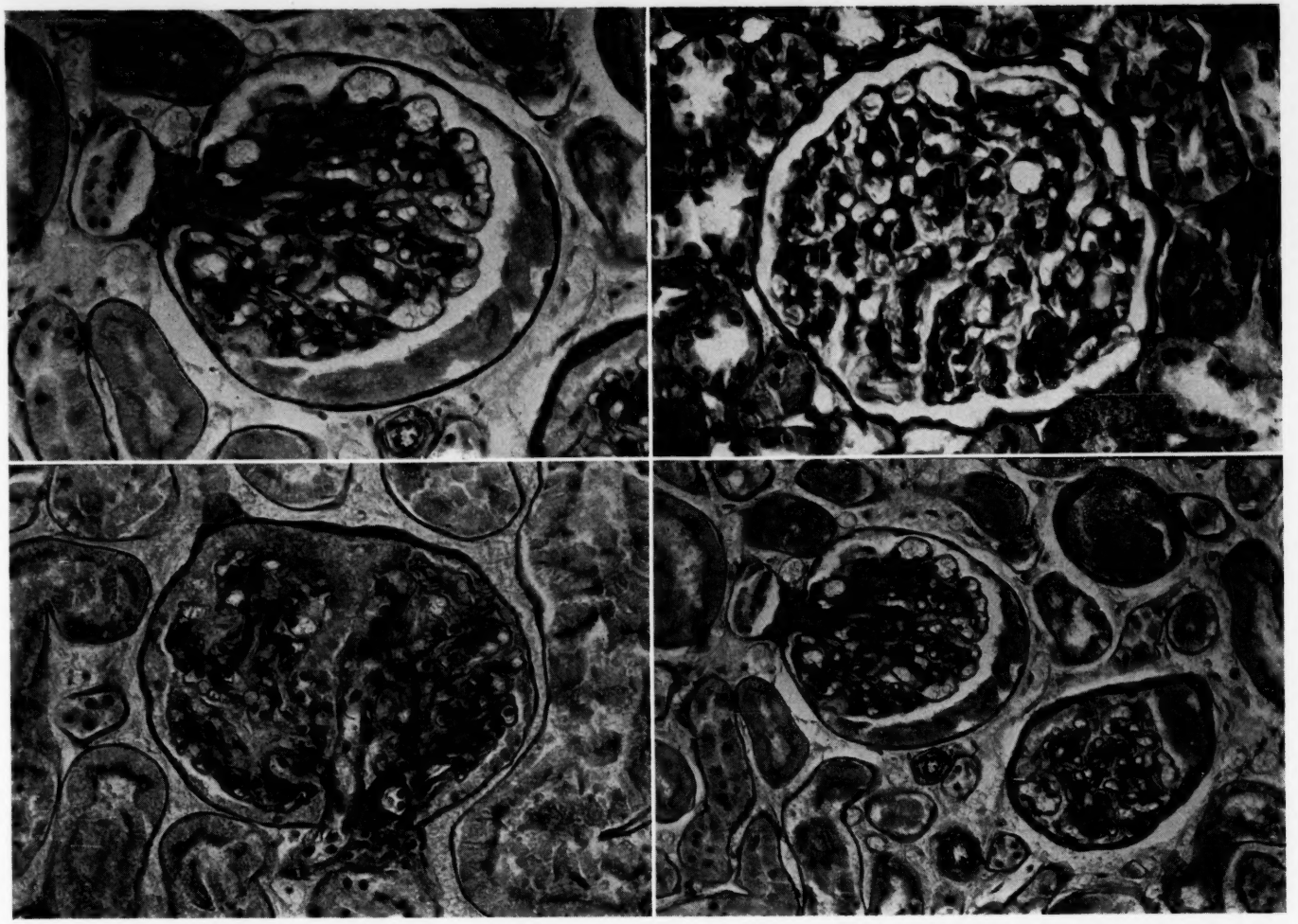


Fig. 2.—*A*, Photomicrograph of glomerulus in kidney biopsy on Dog 3 (×340), taken after 7 months of hydralazine administration. Section shows thickening of basement membrane and the "wire loop" effect. *B*, Photomicrograph of post-mortem kidney sections of Dog 6 (×180), which expired after 3 months of hydralazine administration. Autopsy done within 12 hours of death. Section demonstrates the thickened membranes and almost aneurysmal dilatations in the "wire loop" changes. *C*, Same as *B*, but under greater magnification (×300) to demonstrate the "wire loop" effect. *D*, Photomicrograph of a 24-hour post-mortem kidney section (×300) on a normal dog sacrificed and allowed to remain under environmental conditions similar to those under which Dog 6 was subjected after death. This section demonstrates that post-mortem changes simulating a "wire loop" effect were minimal, even after autolysis was allowed to progress twice as long as in the case of Dog 6. Note the tubular degeneration resulting. (All sections were prepared with PAS stain.)

in the series which has had several convulsive seizures and remained alive. Dogs 6 and 7 expired following convulsions. Dog 3, having undergone eight months of drug feeding, appears to have become somewhat stronger at the time of writing.

Laboratory studies are shown in Table I. Leukocyte and hemoglobin determinations on No. 2 dropped from 9,000 per cubic millimeter and 17.8 Gm. per 100 ml. to 2,000 and 8.0, returning toward prior values upon cessation of the drug. The third dog exhibited serum albumin levels of 3.2 and 3.7 Gm. per 100 ml. and globulin of 4.2 and 4.3 Gm. per 100 ml. The mean albumin of 10 normal dogs was 3.6 (with a standard deviation of ± 0.33); the mean globulin was 2.2 (± 0.27). This change in globulin therefore appears significant. Initial cephalin-cholesterol flocculations were 4+ in all bloods drawn, apparently a normal finding in dogs. The first positive hematological findings were demonstrated on the first animal. All L-E preparations using the dog's own leukocytes taken after three weeks' feeding revealed hematoxylin-staining bodies in large numbers but no typical L-E cells; similar bodies were demonstrated in the second and third dogs as well, done by the method of Zimmer and Hargraves. Dr. Hargraves[14] kindly examined two of these slides and believed that the hematoxylin bodies were consistent with disseminated lupus. Although he found no typical L-E cells, the bodies had the typical homogeneous appearance characteristic of L-E cells. With the use of human leukocytes and dog's serum as described previously, typical L-E cells as defined by Hargraves were found three times in the case of the second dog given hydralazine for three months (Fig. 1). These were sharply differentiated from "tart" cells. The drug was discontinued for three weeks and attempts to demonstrate the L-E cell were unsuccessful. L-E cells were found only after resumption of therapy for an additional five weeks. Similarly, the L-E test was positive in the third animal after four months of drug feeding, became negative when hydralazine was discontinued for one month, then positive after two additional months' feeding. L-E cells were demonstrated in a fourth animal after four months' feeding, while in Dogs 5 to 8 the serum became L-E positive after only two months. Controls run simultaneously with each L-E preparation were negative. Serum cholesterol levels fell moderately during drug administration in 3 of 6 animals.

The serum of the untreated normotensive dog produced an electrophoretic pattern with increased bands of beta and gamma globulins as compared to that of man. Patterns reflecting increased beta, gamma, and alpha$_2$ globulins but consistently decreased alpha$_1$ fractions appeared after the drug was given to dogs.

Microscopic examinations included four renal biopsies, one taken after four weeks and three after four months of drug feeding, and three post-mortem examinations made 2 to 12 hours after death. Sections of the first two post-mortem examinations showed fibrinoid degenerations and prominence of the juxtaglomerular apparatus. These changes were not found in control sections made at comparable periods after death, although the autolysis which

Section demonstrates the thickened membranes and almost aneurysmal dilatations in the "wire loop" changes. *C*, Same as *B*, but under greater magnification (×300) to demonstrate the "wire loop" effect. *D*, Photomicrograph of a 24-hour post-mortem kidney section (×300) on a normal dog sacrificed and allowed to remain under environmental conditions similar to those under which man even after autolysis was allowed to progress twice as long as in the case of Dog 6. Note the tubular degeneration resulting. (All sections were prepared with PAS stain.)

TABLE I. EFFECT OF HYDRALAZINE IN DOGS

DOG NO.	DAILY DOSE PER MG.	CLINICAL SIGNS	WT. (LB.)	W.B.C. PER CU. ML.	HG. PER GM. PER 100 ML.	CHOL. (MG.)	"L. E." PREP.	ELECTROPHORESIS
Dog 1								
Initial	200	Normal and healthy	34	9,700	16.6	78	Neg.	Normal
1 Month	200	Decreased appetite, listless, weak	27	6,200	11.8	59	Hematoxylin bodies*	
6 Weeks	400	Convulsing	22					Increased β, γ, α_2; Decr. α_1
	200	Expired 2 days						
Dog 2								
Initial	200	Normal and healthy	37	9,000	17.8	107	Neg.	Normal
1 Month	200	Decreased appetite, listless, weak				91		
2 Month	200	Eating intermittently, appears ill	29					
3 Month	0	More weakness, coat much more gray	24	2,000	8.0	66	Pos. 3x	Increased β, γ, α_2; Decr. α_1
						67		
3.7 Month	200	Increased appetite, alert and active	27	8,900	14.5	86	Neg.	
5 Month	200	Same as first month	23	4,800	12.0		Pos. 2x	
5.5 Month	200	Expired						
Dog 3								
Initial	200	Normal and healthy	26	11,200	17.5	128	Neg.	Normal
1 Month	200	Decreased appetite, listless	22				Neg.	
4 Month	0	Improved	20	8,400	13.7	101	Pos. 2x	Increased β, γ, α_2; Decr. α_1
5 Month	200	Same as first month	26	8,800	16.4	107	Neg.	
7 Month				7,300		74	Pos.	
8 Month							Pos.	

Dog 4						
Initial	200	Normal and healthy	23	111	Neg.	Normal
1 Month	200	Few convulsions		83	Neg.	Increased β_2,
2 Month	200	More convulsions		106	Neg.	γ, α_2;
4 Month	200	Weak, loss appetite			Pos.	Decr. α_1
Dog 5						
Initial	200	Normal and healthy	23	128	Neg.	
1 Month	200	Weak, no appetite		117	Neg.	
2 Month	200	Exaggerated		100	Pos.	
4 Month	200	Same			Pos.	
Dog 6						
Initial	200	Normal and healthy	27	127	Neg.	
1 Month	200	Weak, appears ill		135	Neg.	
2 Month	200	Loss of appetite		112	Pos.	
3 Month	200	Convulsing; expired			Pos.	
Dog 7						
Initial	200	Normal and healthy	33		Neg.	
1 Month	200	Weak, ill, loss of appetite			Neg.	
2 Month	200	Expired			Pos.	
Dog 8						
Initial	200	Normal and healthy	25		Neg.	
1 Month	200	Weak, loss of appetite, convulsing			Neg.	
2 Month	100	Appears somewhat stronger			Pos.	

*Method of Zimmer and Hargraves only.

had occurred may be partly responsible for these findings. The four-week biopsy on Dog 2 showed only prominence of the juxtaglomerular apparatus. The three renal biopsies taken after four months of feeding of the drug revealed acidophilic hyaline thickening of the basement membrane of the glomerular capillaries, producing the typical "wire loop" effect (Fig. 2). In sections from the animal having expired in the terminal stage of the syndrome, the "wire loop" effect was even more striking, almost aneurysmal in character, and involved almost all of the glomeruli.

DISCUSSION

Clinically, these animals developed signs and symptoms of severe illness. The generalized weakness, lassitude, and weight loss, probably secondary to loss of appetite, are in themselves not manifestations peculiar to the collagen-like syndrome in man. The convulsive seizures are not like any effects recorded in man, but we have produced identical seizures in rats. When the dose of drug was lowered, seizures did not appear. The same reaction has been seen by others.[15] Similar episodes may be produced in animals by thiosemicarbazide which contains the hydrazide configuration. No attempt was made to demonstrate fever nor was arthritis found; the inability to stand was believed to be due to weakness. The drop in serum cholesterol values is not considered part of the syndrome, occurring in 88 per cent of patients receiving hydralazine.[16] Compatible with the collagen-like syndrome and comparable to the human counterpart is the leukopenia accompanied by anemia seen in one animal. This hematological change was coexistent with the appearance of the L-E cell in the peripheral blood and reversed on cessation of drug administration, only to recur on resumption of the drug; however, leukocyte counts in dogs are quite variable. Cephalin-cholesterol flocculations were of no value to the study, since all dogs as well as other laboratory animals have a normal value of 4+. The L-E cells demonstrated revealed in many instances a slightly granular appearance to the inclusion bodies, which is compatible with the findings of Carrera and associates. The clinical microscopist, unaware that the preparations consisted of crossed dog serum and human white cells, read the slides as "positive for cells resembling L-E cells." Controls repeatedly failed to reveal these same cells.

Berman and co-workers[16] were the first to publish methods for the production of L-E cells by positive human serum and leukocytes from the bone marrow of several animals. Although Häserick[17] had been using a similar technique with dog marrow since 1949, Sundberg[18] was the first to report this phenomenon. Carrera and associates were able to cause the reaction with human serum and leukocytes from the buffy coat of the peripheral blood of various animals. To our knowledge, the present experiments are the first to show the L-E factor in dog serum reacting with normal human leukocytes.

Distinctive changes were produced in electrophoretic patterns of plasma proteins. The pattern of dog serum is similar to that of man but with a

slightly wider gamma globulin band. Hydralazine feeding alone in absence of the "collagen-like" syndrome produces a rise in the beta and alpha$_1$ globulins in both man and dog serum. The depression of the alpha$_1$ fraction with noticeable increase in beta and alpha$_2$ globulin is consistent with the findings of Miller and associates[8] who reported on beta and alpha$_2$ globulins in patients with the lupuslike syndrome. Gamma fractions were difficult to evaluate due to the higher values in the normal dog.

The pathologic changes in the kidneys are consistent with those of disseminated lupus erythematosus in that the pathognomonic "wire loop" lesions were found in biopsies and autopsies. The juxtaglomerular apparatus appeared quite enlarged in all sections, but this apparent change was difficult to evaluate in view of the known variation in size of this organ in normal dogs. The liver of the one animal and spleen of the other were considered borderline in enlargement. In man, about 20 per cent of those having this syndrome exhibit hepatomegaly and splenomegaly. From the data shown and from observation, it is my impression that even though all dogs were affected, there was considerable individual variation in reaction to the drug, as regards onset, duration, and severity of the syndrome.

Little as yet is known concerning the mechanism of action and excretion pathways of hydralazine hydrochloride, although considerable work has been done on its general chemical and pharmacologic properties.[19, 20] From this brief study, however, it appears that late toxic reactions can be induced in dogs, with hematological and pathologic findings resembling those found in man as manifestations of both hydralazine toxicity and true collagen disease. Because of the similarity of "hydralazine disease" to that of disseminated lupus, it is possible to utilize this method as an approach to the study of collagen disease. Attempts at inhibition and reversal of this syndrome are in progress.

SUMMARY

Hydralazine hydrochloride (Apresoline), a vasodilator drug used in treatment of arterial hypertension, was orally administered to eight apparently healthy normotensive dogs in an attempt to reproduce the collagen-like syndrome found in man as a delayed toxic reaction to this drug. Doses approximated the maximum used in treatment of human arterial hypertension. The animals developed a syndrome manifested by weakness, weight loss, anemia, and altered electrophoretic patterns of serum proteins; leukopenia, hyperglobulinemia, and hypocholesterolemia occurred in one or more. The L-E cell was demonstrated in the peripheral blood by special techniques in seven animals and hematoxylin bodies in the other. Pathologic specimens of the kidneys revealed changes consistent with those found in disseminated lupus erythematosus.

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STUDIES ON HEMOGLOBIN E

I. The Clinical, Hematologic, and Genetic Characteristics of the Hemoglobin E Syndromes

Amoz I. Chernoff, M.D., Virginia Minnich, M.S., St. Louis, Mo., Supa Na-Nakorn, M.D., Soodsarkorn Tuchinda, M.D., Channivat Kashemsant, M.D., Bangkok, Thailand, and Renate R. Chernoff, B.S.C., St. Louis, Mo.

IN 1954, Minnich and co-workers[1] reported the occurrence of a severe form of thalassemia in thirty-two individuals in Thailand. Except in minor respects, the clinical and hematologic characteristics of these cases were indistinguishable from those described in numerous reports of thalassemia major emanating not only from the Mediterranean area but also from the United States. However, the somewhat milder course of the disease as it occurred among many of the Thais and the number of adult individuals apparently afflicted with this disorder were different from observations usually made in cases of Cooley's anemia. Genetic studies, furthermore, proved most difficult to reconcile with a diagnosis of true Cooley's anemia; although one parent usually had hematologic findings compatible with thalassemia minor, similar abnormalities were often lacking in the second parent. Because of these observations, it was suggested that (1) factors other than the gene responsible for thalassemia may play a role in the pathogenesis of the anemia and (2) the interaction of a single gene for thalassemia with a gene for an abnormal hemoglobin must be considered.[1] Analogous situations are recognized in the combination of Hgb S or Hgb C with the gene for thalassemia, where the resulting syndromes differ somewhat from thalassemia major in a manner not unlike that observed in the cases reported from Thailand.[2, 3, 4]

With the cooperation of members of the departments of pediatrics and medicine, Siriraj Hospital, Bangkok, blood specimens from some of the patients previously reported were sent to St. Louis in order that their hemoglobin types might be studied. Electrophoretic analysis indicated the presence of a new hemoglobin form, Hgb E, in the blood of several of these specimens. A report of these observations has already been published.[5] Further study

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo., and The Departments of Internal Medicine and Pediatrics, Siriraj Hospital, Bangkok, Thailand.

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was carried out in Bangkok during the second half of 1954; the following communication summarizes the results of our investigations of the clinical, laboratory, and genetic features of this new hemoglobin type.

Throughout this report, the terms thalassemia major and minor will be used in preference to Mediterranean anemia and trait or any of the many other names assigned to the thalassemia syndromes. The reasons for, as well as the objections to, the use of the designation thalassemia have been extensively reviewed by Powell and associates[6] and by Dameshek[7] and need not be repeated here.

METHODS AND MATERIALS

Routine hematologic studies were carried out by standard methods as previously described.[1] Fecal urobilinogen determinations were based on a modification of the method of Watson[8] and the hemolytic index was calculated by use of the formula of Miller, Singer, and Dameshek.[9] The Coombs test was performed with commercial antiglobulin sera in the usual manner, but the results were often questioned because of the number of apparently false positive tests among normal individuals. These questionable reactions may have been due to (1) the age of the antisera and (2) repeated thawing and freezing of the material as a result of almost daily temporary failures of electrical power and refrigeration. The technique of Daland and Castle,[10] employing sodium metabisulfite, was used for determining the presence of sickling.

Paper electrophoresis was carried out as previously described,[5] but at a somewhat lower voltage, generally for a period of 5 to 6 hours per run. Since large fluctuations of line voltage often took place, no attempt was made to standardize the time and the electrophoretic runs were interrupted when separations, sufficient for interpretation, were apparent.

The amount of fetal hemoglobin was determined by the alkali denaturation procedure modified for use on the Evelyn colorimeter.[11]

Clinical material was drawn from the large number of patients seen in the departments of medicine and pediatrics of the University of Medical Sciences, Siriraj Hospital, Bangkok, Thailand, and included both hospitalized and outpatient subjects. In addition, a number of normal medical students and nurses with the Hgb E trait cooperated in these studies. Careful histories were obtained from many of the patients reported. Complete physical examinations were done when possible. Only those individuals on whom adequate laboratory information was accumulated were used in evaluating the effects of Hgb E.

RESULTS

Characteristics of Hemoglobin E.—The chief characteristics of Hgb E have been described in a previous publication.[5] The essential distinguishing feature is a mobility on paper electrophoresis at pH 8.8 (veronal buffer, ionic strength 0.06) which is greater than Hgb C but less than that of Hgb S (Fig. 1). Independently, Itano and co-workers[12] described a hemoglobin type in a patient with an atypical hemolytic anemia which behaves on paper electrophoretic analysis in an identical fashion. Exchange of samples of the two Hgb E specimens indicated similar migration patterns at pH 8.8 or 9.0 on paper, but there has been no opportunity as yet to study their relative mobilities in the Tiselius apparatus at pH 6.5. In view of the similarities between the clinical and hematologic abnormalities in the patient with thalassemia-Hgb E described by Sturgeon and associates[13] and those recorded in

this communication, there seems to be little doubt that the two pigments are the same. Definite information on this question will, however, require the more critical test of moving boundary electrophoresis.

Hgb E is denatured by alkaline reagents in a manner similar to Hgb A.[5] Solubility studies and ultraviolet and visual absorption spectra determinations reveal no differences from the normal adult compound, Hgb A.[5, 12] Finally, red cells containing Hgb E do not sickle.

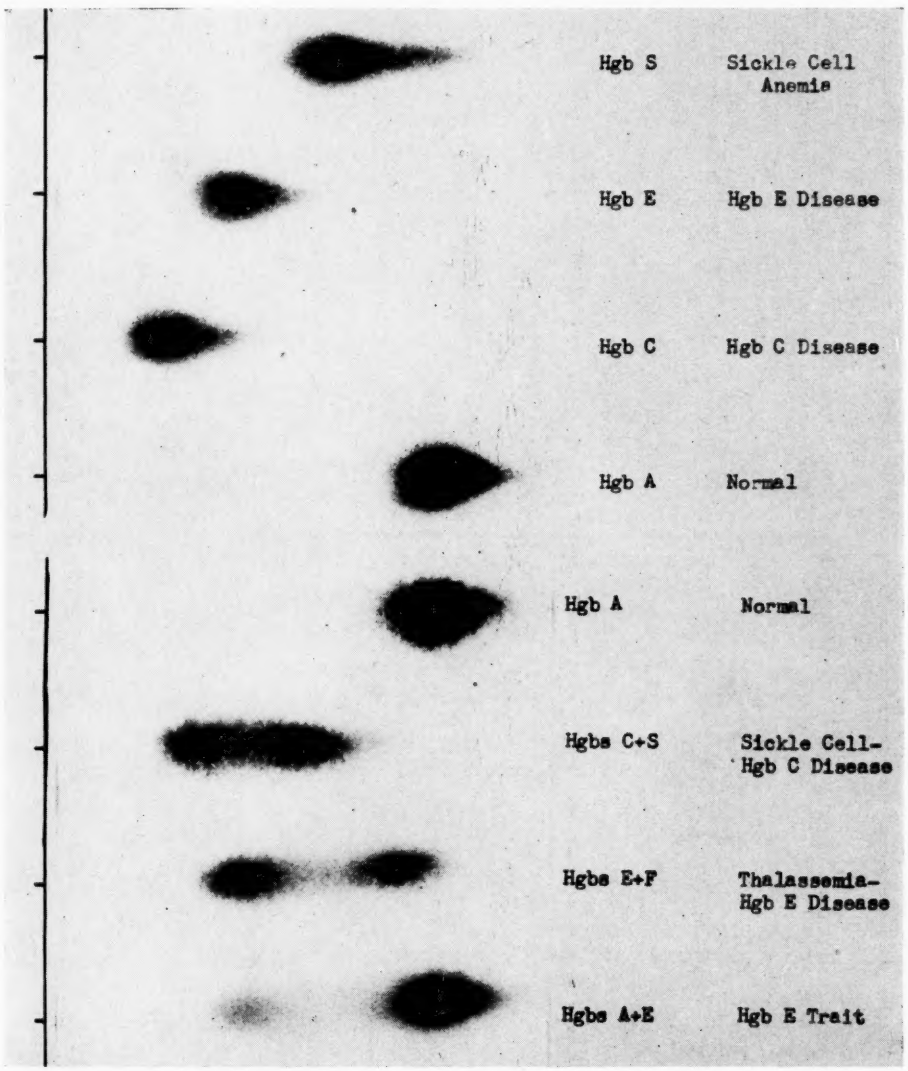


Fig. 1.—Paper electrophoresis of Hgb E at pH 8.6 (veronal buffer, ionic strength 0.06). The upper portion compares a specimen from a patient with homozygous Hgb E disease with specimens obtained from other homozygous hemoglobin diseases. The lower portion contains specimens from patients with Hgb E trait and thalassemia-Hgb E disease.

Hemoglobin E Trait.—Thirty-six individuals whose blood contained Hgb A plus Hgb E were available for detailed study (Table I). Genetic studies,

TABLE I. CLINICAL AND LABORATORY DATA IN THE HGB E TRAIT

CASE	MEMBER FAMILY GROUP	RELATIONSHIP	AGE	SEX	RBC	Hgb	PCV	MCV	MCHC	RETICS	TARGET CELLS	OSMOTIC FRAGILITY	I.I.	VdB	Hgb F	SPLENOMEGALY	HEPATOMEGALY	REMARKS
1	-	-	20	F	6.02	14.7	48	80	31	0.6	<0.5	0.40-0.32	-	-	N	0	0	
2	-	-	20	M	6.95	15.4	50.5	72.5	30	-	-	0.39-0.32	-	-	N	0	0	
3	-	-	21	M	5.91	14.5	46	74	33	0.9	<2.0	0.36-0.29	5	-	N	C.M.	1.3	
4	-	-	21	M	5.45	14.6	45.5	84	32	0.6	0.0	0.39-0.29	10	-	N	0	0	
5	-	-	21	F	5.65	13.0	40	71	33	0.8	-	0.40-0.32	5	-	N	C.M.	C.M.	
6	-	-	21	F	6.11	15.3	48	78	32	-	-	-	-	-	-	-	-	Malaria age 11
7	-	-	22	F	4.76	12.6	40	84	32	1.3	<1.0	0.38-0.30	10	-	N	0	0	Amoebic dysentery
8	-	-	22	M	5.7	16.0	48	84	33	0.6	<1.0	0.38-0.30	10	-	N	0	0	
9	-	-	23	M	4.79	14.7	47	98	31	0.4	<0.5	0.41-0.32	8	0.25/0.73	N	0	0	11% eosinophiles
10	-	-	-	F	3.5	10.1	31	88	33	0.2	1.0	-	-	-	N	-	-	
11	-	-	46	F	3.92	10.4	32.5	83	32	-	-	0.39-0.34	2	-	N	-	-	Bone marrow normal
12	-	Daughter (Case 13)	35	F	4.35	11.1	36	83	30	1.6	<1.0	0.37-0.31	5	-	-	0	C.M.	
13	-	Mother (Case 12)	63	F	4.97	11.4	36	72	32	0.8	-	-	5	-	-	-	-	
14	A	Mother	-	F	4.50	11.4	34	76	33	-	5.2	0.40-0.22	5	0.19/1.14	N	-	-	
15	B	Father	44	M	5.0	13.8	42.5	85	32	0.2	0.2	0.38-0.26	5	0.24/0.64	N	-	-	
16	C	Father	35	M	5.78	15.3	44.5	77	34	1.9	4.0	0.39-0.28	7.5	0.09/0.13	N	0	C.M.	Bone marrow normal
17	D	Mother	-	F	4.32	11.4	34	78	33	0.4	0.0	0.48-0.30	5	0.19/1.80	N	-	-	
18	E	Mother	-	F	4.43	13.9	37.5	80	37	0.4	0.0	0.46-0.32	7.5	0.13/0.43	N	-	-	
19	F	Mother	-	F	5.43	14.3	42.5	78	33	0.6	0.0	0.46-0.32	10	0.25/0.64	N	-	-	
20	H	Brother	10	M	4.22	12.0	36	85	33	0.4	3.5	0.41-0.30	2	-	N	0	1.0	
21	J	Father	43	M	5.55	16.5	48	87	34	1.4	3.0	0.41-0.32	9	-	N	0	0	Bone marrow E:M 1:1

22	L	Mother	–	F	4.26	13.0	37	86	35	0.2	0.0	0.40-0.30	5	–	N	–	–	
23	N	Father	–	M	5.26	15.8	43.5	83	36	0.6	<1.0	0.38-0.28	5	0.34/0.98	N	–	–	
24	O	Father	–	M	4.23	12.6	38.5	91	32	0.4	0.0	0.44-0.36	5	0.32/0.73	N	–	–	
25	S	Mother	65	F	4.06	11.0	34	82	32	0.2	0.2	0.40-0.32	7.5	–	N	–	–	
26	T	Mother	50	F	4.08	12.3	38.5	94	32	–	–	0.38-0.28	5	–	N	0	0	
27	W	Father	57	M	5.10	14.4	45	88	32	–	3.4	0.38-0.28	5	–	N	0	1.5	
28	Z	Father	–	M	6.06	15.5	46	75	34	0.4	0.0	0.42-0.24	5	0.32/0.64	N	–	–	
29	BB	Brother	24	M	5.52	14.3	44	79	32	0.6	0.0	–	2	..	N	0	0	
30	CC	Mother	–	F	4.65	12.0	36.5	78	32	0.3	–	0.48-0.30	–	0.22/0.51	N	–	–	
31	–	–	54	M	2.43	8.1	24	99	34	1.6	14.0	–	7.5	0.25/1.25	N	1.0	3.0	Pneumonia, diabetes
32	–	–	54	M	3.94	10.1	35.5	91	28	0.4	–	0.35-0.27	10.0	0.72/1.80	N	C.M.	6.0	Ca of liver (?)
33	–	–	–	M	2.05	3.2	15	73	21	0.8	–	–	10.0	–	N	–	–	Fe deficiency
34	C	Sister	9	F	4.79	12.5	37	87	34	0.5	2.4	0.37-0.25	7.5	0.08/0.26	N	0	1.5	Posthepatitis
35	I	Mother	30	F	4.13	8.0	28	69	29	0.1	25.0	0.37-0.22	2	–	N	C.M.	3.5	17% eosinophiles (?) Fe deficiency
36	K	Mother	–	F	2.63	8.1	24	91	34	0.6	0.5	0.48-0.32	5	0.22/0.28	N	–	–	(?) Blood loss

Symbols used in this and subsequent tables: Age in years; RBC = red cell count per cu. mm. × 10^6; Hgb in Gm. per 100 ml.; PCV = packed cell volume in per cent; MCV = mean corpuscular volume in cu. microns; MCH = mean corpuscular hemoglobin in $\mu\mu$; MCHC = mean corpuscular hemoglobin concentration in per cent; WBC = white count per cu mm. × 10^3; retics = reticulocytes in per cent; platelets per cu. mm. × 10^3; NRBC = nucleated RBC per 100 WBC; target cells in per cent; stippling = basophilic stippling of erythrocytes; osmotic fragility: first figure refers to concentration of saline with 10% hemolysis, second figure 90% hemolysis; I.I. = icterus index; VdB = van den Bergh in mg. per cent, immediate reacting bilirubin in numerator, total in denominator; Hgb F in per cent. N = normal amount; H.I. = hemolytic index; splenomegaly and hepatomegaly in cm. below the costal margin; race based on origin of the four grandparents; x-ray findings listed as positive when suggestive of findings in severe hemolytic anemias; E:M = erythrocytic-myelocytic ratio; C.M. = costal margin; O = absent; – = no information; + and ++ = degree of abnormality; Th-E Dis. = thalassemia – Hgb E disease.

to be mentioned below, suggest that the pattern of Hgb A plus Hgb E represents the heterozygous state of Hgb E and therefore may properly be referred to as the Hgb E trait. Although no quantitative determinations were carried out on the relative amounts of Hgb A and Hgb E, gross inspection of the paper electrophoresis strips always revealed a darker spot of hemoglobin in the position of Hgb A (Fig. 1). Analogous findings are recorded in all other known heterozygous hemoglobin trait states, where Hgb A invariably forms the major pigment type.[14]

Thirty of the individuals with the Hgb E trait were apparently free of other incidental diseases. Some of these thirty subjects had a variety of minor complaints, but they could not be exhaustively studied and no definite

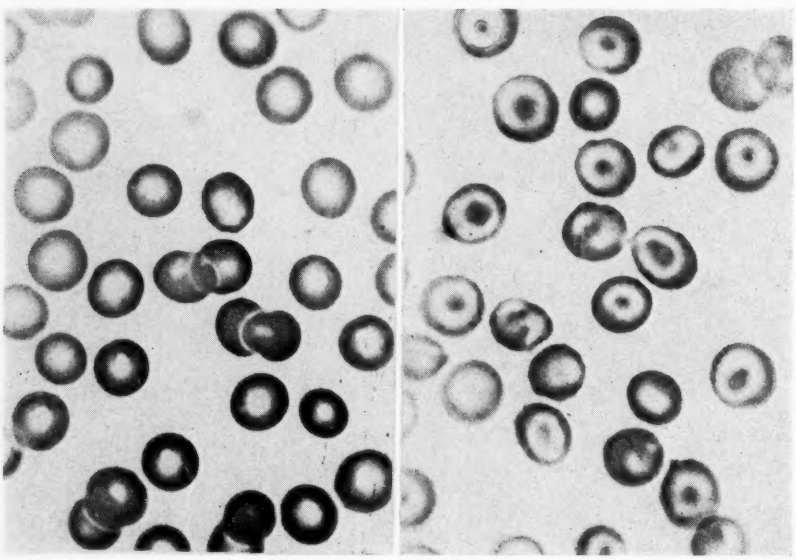


Fig. 2.—Stained peripheral blood film of an individual with Hgb E trait (left) and of a patient with homozygous Hgb E disease (right) (×1,200; reduced ⅛).

diagnoses were evident. In view of the high rate of parasitism and the incidence of hepatic disturbances and iron deficiency among many of the inhabitants of the Orient, it was not surprising to find a number of patients with Hgb E trait with abnormal hematologic findings. Inspection of the data from those appearing healthy, however, suggests that the Hgb E trait is unassociated with either physical disability or hematologic abnormality. No consistent history of anemia, cardio-respiratory distress, jaundice, leg ulcers, or arthralgias could be obtained. Physical examinations were unremarkable except when incidental disease processes were present.

A summary of the hematologic data obtained from patients with the Hgb E trait is presented in Table I. Except for a tendency to slightly microcytic normochromic erythrocytes, routine studies were within normal limits. Examination of the stained blood films (Fig. 2) revealed no morphologic abnormalities except for an occasional red cell with very fine basophilic stippling, seen in

the specimens of several of the patients. The osmotic fragility curves were within normal limits in 10 of the 26 subjects and moderately shifted to the right in the remaining 16. What role associated conditions like iron deficiency anemia, liver disease, and others played in this finding could not be ascertained from our data. Sickling of the erythrocytes could not be elicited, and no instance of Hgb S in Thailand was observed. Bone marrow examinations, carried out in three of the patients, and hemolytic index determinations, calculated for three other individuals, revealed no evidence of increased red cell turnover. Coombs tests were usually negative. In two completely asymptomatic healthy subjects, weakly positive antiglobulin tests were obtained on one occasion. Whether these represented technical errors or resulted from the use of deteriorated or contaminated sera cannot be stated, but no interpretation of a weakly positive Coombs test was felt to be justified under these circumstances. In several instances, roentgenograms of the bones were available and did not reveal alterations generally associated with the hemolytic syndromes.

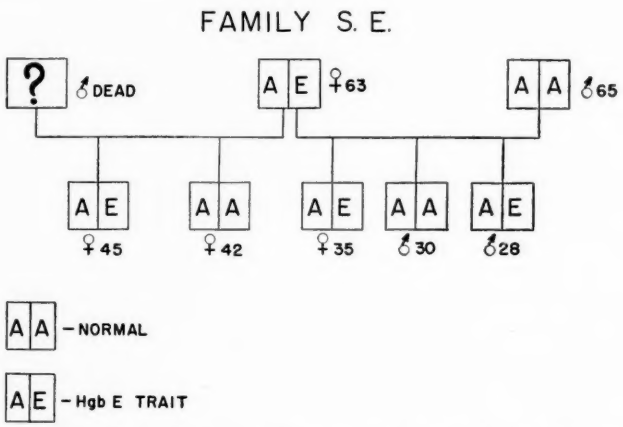


Fig. 3.—Genetic representation of Family S. E. harboring the gene for Hgb E.

Six patients with Hgb E trait had associated diseases which caused a variety of hematologic changes. Most notable were those associated with liver pathology in which elevations of target cell counts, decreases in osmotic fragility of the erythrocytes, and changes in the van den Bergh determinations were observed. None of the hematologic abnormalities noted in this group was thought to be primary. In addition to the presence of Hgb A and Hgb E on paper electrophoresis (Fig. 1), hemoglobin analysis showed no increase in the amount of Hgb F by the alkali denaturation technique.

Complete genetic studies could be performed in only a few of the patients described. Whenever both parents of an individual with the Hgb E trait were examined, the gene for the abnormal hemoglobin could always be detected in at least one of them. In all probability, Hgb E is an allele of the normal adult pigment. It would seem, therefore, that the hereditary pattern of the

TABLE II. CLINICAL DATA IN PATIENTS WITH THALASSEMIA-HGB E DISEASE

CASE NO.	MEMBER OF FAMILY GROUP	AGE	SEX	RACE OTHER THAN THAI	NO. OF SIBLINGS			ONSET OF MAJOR SYMPTOMS (YEARS)	SPLENO-MEGALY	HEPATO-MEGALY	X-RAY FINDINGS	TRANS-FUSION THERAPY	REMARKS
					LIVING	DEAD	WITH PROBABLE Th-E DIS.						
1	A	1¾	F	-	3	0	1	Birth	At CM	3.3	-	425 ml.	
2	A	8½	M					4	Absent	3.3	Positive	1,225 ml.	Splenectomized, 1953
3	B	1⅓	M	Chinese 1/4	4	1	0	1/3	0.0	3.3	Positive	270 ml.	
4	C	1¼	M	-	3	2	2	2/3	3.3	3.3	-	2-3 x	
5	C	3	F					1/2	9.0	5.0	Positive	Repeated	
6	C	12	F					2	>15.0	8.5	Positive	Repeated	
7	D	2¼	F	-	3	2	1	3/4	Below umbilicus	4.5	Positive	300 ml.	
8	E	5	M	-	0	0	0	3	3.3	3.0	Positive	2,400 ml.	Splenectomized, 1954
9	F	5	M	Chinese 2/4	3	1	1	5	3.3	4.5	Positive	150 ml.	Hepatitis
10	G	5	F	-	4	0	0	4	4.0	2.0	Positive	0	
11	H	2	M	-	2	3	3	1	4.5	4.5	-	0	
12	H	11	F					3	To iliac crest	3.5	Positive	Repeated	Splenectomized, 1954
13	I	6	M	-	2	0	1	3	9.0	4.0	-	500 ml.	
14	J	7	M	-	1	1	1	1	5.0	CM	-	2,700 ml.	
15	K	3½	M	-	1	1	1	1	8.5	1.8	Positive	1,000 ml.	
16	L	9	F	Indian 3/4	2	0	0	2	8.5	8.5	Positive	4,750 ml.	
17	M	4	M	-	4	0	1	1/2	15.0	5.0	Positive	Repeated	Splenectomized, 1954
18	N	6	F	-	1	1	0	3	7.0	4.0	Positive	Repeated	Splenectomized, 1954
19	O	12	M	-	3	0	0	3	>20.0	7.0	Positive	Repeated	Splenectomized, 1954
20	P	10	M	Chinese 2/4	0	2	1	5/12	Absent	6.5	Positive	200 ml. since 1950	Splenectomized, 1950
21	Q	9	M	-	4	0	0	4	Iliac crest	4.0	Positive	4,900 ml.	
22	R	15	M	-	5	4	7	1	8.0	4.0	Positive	1,050 ml.	Splenectomized, 1954
23	S	22	F	-	3	1	0	14	10.0	6.5	Positive	1,875 ml.	Splenectomized, 1954
24	T	17	F	-	0	1	0	11	7.5	1.5	Positive	850 ml.	Splenectomized, 1954
25	U	24	F	-	2	1	0	4	14.0	6.0	Positive	700 ml.	
26	V	30	M	-	4	1	0	19	8.5	1.5	Positive	0	
27	W	28	F	-	5	0	0	11	Below iliac crest	2.5	Positive	2,450 ml.	Splenectomized, 1954
28	X	31	M	-	2	3	1	10	12.0	6.0	Positive	3,150 ml.	Splenectomized, 1954
29	Y	27	M	Chinese 3/16	0	0	0	5	7.5	3.5	Positive	0	Splenectomized, 1954
30	Z	11	M	-	2	2	1	7	4.0	2.0	-	700 ml.	Hepatitis
31	AA	19	F	-	0	0	0	?12	Absent	5.0	-	0	Splenectomy, 1949
32	BB	22	F	-	3	0	0	5	8.0	3.0	-	700 ml.	Splenectomy, 1954

See Legend under Table I.

Hgb E trait is analogous to that seen in the Hgb S and Hgb C traits.[14] A typical kinship, Family S. E., harboring the gene for Hgb E is illustrated in Fig. 3 and includes Cases 12 and 13, Table I.

THALASSEMIA-HEMOGLOBIN E DISEASE

Thirty-two individuals believed to have thalassemia-Hgb E disease were extensively studied (Tables II and III). Twenty-eight of the 32 were classified as severe examples of this disease process and four as moderately severe. The marked variability of the symptom complex referred to as thalassemia-Hgb E disease, even within the group classified as severe, is readily apparent.

Severe Thalassemia-Hgb E Disease.—Of the twenty-eight patients in this group, 16 were males and 12 females (Table II). At the time of study, eighteen were 10 years of age or less, nine from 11 to 30 years of age, and one 31 years old. Only four of the twenty-eight were not of pure Thai extraction, when family trees were traced back for two generations or more. Two were one-half Chinese, one was one-fourth Chinese, while one family was three-fourths Indian; the remaining racial background in each case was Thai.

General clinical considerations: Almost without exception, signs of impaired health were noted during the first decade of life and, for the most part, before the age of five. In one child, the onset of the disease could be dated from birth. Only four individuals reported that the first symptoms appeared after the age of ten, but all admitted to having been in poor health for as long as they could remember. The initial complaints varied from patient to patient, and in many instances several symptoms appeared simultaneously. Most common was the development of a mass in the left upper quadrant, which often antedated by a short time the onset of the next most common symptom, that of pallor. In addition, almost every patient complained of most of the following symptoms: shortness of breath, easy fatigability, palpitations, dyspnea on effort, repeated upper respiratory infections, frequent bouts of fever with or without chills, and intermittent jaundice. The jaundice, which varied erratically in severity, was generally accentuated by upper respiratory infections and was accompanied by the passage of dark-colored urine and increased signs of anemia. Chills and fever during these exacerbations, which may represent hemolytic crises, were common. With the passage of time, abdominal enlargement tended to become enormous, whereas the general development of the child was definitely behind that of other Thai children. Appetites were generally poor; failure to gain weight and to grow in stature was reported in all the patients. The ability of the children to sit up, walk, talk, and develop mentally was strikingly impaired. Complaints of sleeplessness, lethargy, listlessness, irritability, and headaches were common. Secondary sexual characteristics, in those individuals old enough to evaluate this factor, were almost absent. Only one of the six females over the age of eleven has menstruated, and in this patient, aged 28, menses have been scanty and infrequent. None of the males over 15 has shaved. Since Thai men characteristically have light beards, this point is difficult to evaluate. One of the two males over the age of 20 said he was the father of two children.

TABLE III. LABORATORY DATA IN PATIENTS WITH THALASSEMIA-HGB E DISEASE

CASE NO.	RBC	Hgb	PCV	MCV	MCHC	WBC	RETICS	PLATE-LETS	NRBC	TARGET CELLS	STIP-PLING	VdB	OSMOTIC FRAGILITY	Hgb F	H.I.	REMARKS
1	2.15	5.3	18	83	29	11.8	2.9	400	11	12.2	–	0.45/1.44	0.48-0.24	85.4	–	
2	2.61	5.7	21	80	27	12.5	11.4	Numer-ous	700	22.8	–	0.25/1.17	0.34-0.10	15.3	–	Postsplenectomy
3	1.3	3.0	9.5	73	32	13.8	1.2	250	6	7.8	++	0.45/1.03	0.36-0.16	24.2	–	
4	2.67	4.4	17	64	26	22.0	7.0	290	46	15.0	++	–	0.42-0.21	22.5	–	
5	2.55	5.33	18	71	30	12.3	6.4	170	14	8.0	++	0.36/1.09	0.40-0.20	13.6	–	Posttransfusion
6	2.00	3.54	15	75	24	11.5	9.5	100	18	0.5	+	1.08/3.16	0.43-0.32	11.3	62	Posttransfusion
7	2.26	4.8	16	71	30	16.0	2.7	190	59	3.8	–	0.60/1.52	0.46-0.28	29.2	–	
8	3.64	7.1	25.5	71	28	8.1	4.3	Many	12	20.0	–	–	0.36-0.22	54.9	–	
9	2.8	6.4	21	75	31	23.0	2.6	420	13	1.6	+++	3.46/7.52	0.50-0.24	43.2	–	
10	2.77	6.3	21	75	30	12.4	2.0	300	36	17.4	–	0.16/1.52	0.48-0.18	31.9	–	
11	3.75	7.3	25	67	29	33.6	3.5	700	15	28.5	0	–	0.34-0.14	25.4	–	
12	3.15	5.3	19	61	28	13.0	7.6	130	10	5.0	++	0.19/1.36	0.44-0.18	24.2	163	
13	2.40	4.3	16	70	27	12.0	4.8	100	5	6.1	+	0.22/1.38	0.41-0.16	29.0	–	
14	3.13	6.6	24	77	28	11.5	2.1	Many	12	10.5	0	0.23/1.94	0.40-0.20	23.0	–	Posttransfusion
15	3.7	6.6	23.5	64	28	12.9	1.6	300	20	2.2	–	0.25/2.31	0.44-0.22	20.0	–	Posttransfusion
16	0.98	2.3	7.5	76	31	9.5	1.0	250	5	5.6	–	0.73/1.73	0.44-0.24	7.9	–	? Aplastic crisis

17	2.21	4.6	15	68	31	7.6	4.6	300	29	7.0	+	0.18/1.60	0.43-0.26	5.5	-	Posttransfusion
18	3.50	8.55	27.5	79	31	7.0	4.3	260	5	7.2	+	0.16/1.68	0.43-0.15	9.9	-	Posttransfusion
19	3.4	7.95	23	69	32	6.0	3.0	160	12	4.8	+	0.13/1.36	0.42-0.27	6.7	93	Posttransfusion
20	3.60	6.92	25	70	28	16.0	5.8	1,000	210	31.0	0	-	0.32-0.10	14.5	-	Postsplenectomy
21	1.65	3.6	12.5	76	29	17.0	5.5	Many	60	4.4	-	0.48/2.08	0.42-0.20	Elevated	-	Posttransfusion
22	2.25	5.2	18.5	82	28	9.6	3.0	430	11	4.2	-	0.13/2.16	0.40-0.28	37.0	-	
23	2.64	5.0	18.5	70	27	6.7	4.0	52	7	12.2	+	0.26/2.25	0.42-0.18	23.2	508	Posttransfusion
24	3.22	6.4	22.5	69	28	6.6	4.3	219	6	44.2	+	0.30/0.68	0.47-0.24	23.6	170	
25	1.75	3.0	11	63	27	4.3	4.0	Many	28	>20.0	-	0.38/1.83	0.44-0.24	27.0	106	
26	1.51	3.4	11	73	31	2.2	4.7	140	0	5.0	-	0.19/1.31	0.46-0.18	5.9	-	
27	3.2	8.4	32	100	26	8.1	0.2	961	296	40.5	-	0.45/3.99	0.38-0.18	22.6	-	Postsplenectomy Posttransfusion
28	2.49	5.1	20	80	27	4.6	1.4	140	13	20.0	-	0.73/1.40	0.40-0.16	18.5	-	Posttransfusion
29	4.18	8.8	29	70	30	9.5	5.5	211	3	40.5	-	0.65/2.65	0.38-0.18	24.7	-	
30	4.10	6.1	21.5	52	28	24.0	2.0	200	16	16.7	++	16.3/36.2	0.36-0.16	6.0	-	Severe hepatitis
31	4.30	9.0	32	75	28	15.0	14.5	Many	296	>20.0	-	-	0.40-<0.20	15.6	-	Postsplenectomy
32	4.17	8.4	30	72	28	9.1	8.2	271	1	21.2	-	0.35/1.68	0.46-0.16	9.1	-	

See legend under Table I.

Less frequent complaints were those of edema of the face and extremities, usually seen during extremely severe exacerbations of the disease, ascites (in two patients), and in several individuals, vague right upper quadrant pain probably related to cholelithiasis (gallstones were found and removed in one patient, Case 17, Tables II and III, at time of splenectomy) or hepatic involvement. Leg ulcers, rarely seen in the thalassemia syndromes, proved to be an infrequent complication of thalassemia-Hgb E disease, only three patients having complained of a single lesion each. Bone, joint, or muscle pains were generally absent. Partial baldness was noted in several of the younger children (Fig. 4). Parasitic, malarial, bacterial, and viral diseases, all extremely common in Thailand, complicated the clinical course of most of these patients at one time or another. Hepatitis of undetermined etiology seemed, however, to be especially common in patients with thalassemia-Hgb E disease, irrespective of the prior use of blood transfusion therapy. Although none of the patients under study died, at least 12 siblings of individuals in this group succumbed with syndromes suggestive of thalassemia-Hgb E disease. Intercurrent infection, usually of the upper respiratory tract, accounted for most of the deaths.

Physical findings:

General Appearance.—All patients with the severe form of thalassemia-Hgb E disease appeared chronically ill and many presented a striking body habitus (Fig. 4). Particularly in the children, the small stature, wasted extremities, and poor body development stood out in marked contrast to the huge protuberant abdomen, which completely dominated their physical appearance. So great was the abdominal enlargement that many children five and six years of age found it necessary to arch their backs to maintain their balance and the erect position. In many ways this stance resembled that of a pregnant woman. The abdominal enlargement, coupled with the generalized wasting, dried, wrinkled skin, sunken eyes, and wizened expression frequently suggested a diagnosis of progeria.

Facies.—Since Mongoloid facies have often been reported as part of the thalassemia syndromes,[1, 15, 16] it was of some interest to evaluate the presence of abnormal facies in this group of patients. Normal Thais have minor facial characteristics, common in the Orient, which form part of the typical Mongoloid facies, such as small epicanthal folds and a somewhat broadened base of the nose. Consequently, it is difficult to be sure where the normal changes cease and Mongoloid features begin. No instance of obvious Mongoloid facies was encountered in the 30 patients in this group, although a number of individuals had very suggestive changes. Nevertheless, the facial appearance of our patients resembled in many ways the Mongoloid facies described in instances of thalassemia from the Mediterranean area, where oriental features do not normally complicate the picture.

Skin, Mucous Membranes, and Conjunctivae.—Icterus and pallor were usually moderate in degree, but the actual intensity of these findings paralleled the abnormalities of pigment metabolism as detected in the laboratory. Three

patients with intense jaundice were suffering from infectious hepatitis. No active leg ulcers were seen, but scars on two of the patients indicated previous lesions of this type.

HEART.—The heart was generally enlarged and systolic murmurs were frequently present. Cardiomegaly was roughly proportional to the severity of the anemia and in all cases was felt to be secondary to the low hemoglobin level.

LYMPH NODES.—Generalized enlargement of peripheral lymph nodes was noted in most of the subjects under study. Nodes were usually small, less than 1.5 cm. in diameter, and nontender.

LIVER.—Hepatomegaly was present in every case, the lower edge of the liver being palpated from just at the costal margin to as low as 8.5 cm. below the right costal margin (Fig. 4). The greatest degree of hepatomegaly was

Fig. 4.—Front and side views of children with thalassemia-Hgb E disease. From left to right: Patients 17, 18, 14, 19, Table II.

encountered in the older, more severely affected patients who had received multiple transfusions. The liver was usually smooth, moderately firm, and only occasionally tender.

SPLEEN.—Splenomegaly was detected in 25 of the 26 patients with severe thalassemia-Hgb E disease who had not been subjected to splenectomy at the time they were first examined. The only individual without a palpable spleen was a 15-month-old child with typical features of thalassemia-Hgb E disease (Case 3, Table II). Rarely was the spleen felt less than 5 cm. below the left costal margin, and frequently the lower pole extended below the level of the umbilicus and even into the pelvis itself (Fig. 4). The smallest spleens tended to be found among the youngest patients, but no correlation existed between the actual degree of organ enlargement and the state of the disease. The spleen was almost always firm, at times to the point of hardness, grossly irregular, especially in the regions of the notch, and nontender.

Hematologic Studies.—In general, the hematologic data of patients with thalassemia-Hgb E disease were indistinguishable from the findings in severe examples of Cooley's anemia (Table III). The anemia may be characterized as a severe microcytic hypochromic hemolytic process. Red cell counts averaged from 1.5 to 3.5 million per cubic millimeter, with the majority of values in the range of 2.0 to 3.0 million per cubic millimeter. Hemoglobin levels almost invariably fell within the range of 3 to 7 Gm. per 100 ml., except in those patients who had been transfused. The lowest value noted, 2.3 Gm., was in a patient with less than 1.0 million red cells per cubic millimeter, during a probable aplastic crisis.[17] The mean corpuscular volume (MCV) generally fell between 65 and 75 c.μ. Occasional exceptions were seen in patients with severe hepatitis and in individuals studied in the postsplenectomy and posttransfusion states. Mean corpuscular hemoglobin concentrations (MCHC) averaged from 27 to 29 per cent. Higher values occurred, in almost every instance, in individuals who had recently received blood transfusions.

Reticulocyte counts were moderately elevated, with most of the values falling between 4 and 6 per cent. When superimposed infections were prominent, the reticulocyte count tended to drop to normal. Nucleated red cells (NRBC) were seen in the peripheral blood films of all but one patient. Usually, in the nonsplenectomized patients, from 10 to 50 NRBC per 100 WBC were present. As many as 700 NRBC per 100 WBC appeared during the postsplenectomy state. Target cell counts ranged from few to many and, in general, comprised from 5 to 25 per cent of the erythrocytes on the stained blood film (Fig. 5). As was to be expected, the greatest numbers of target cells were seen in patients with hepatitis and those studied following splenectomy. Sickle cell tests were invariably negative.

The erythrocyte morphology was characterized by marked aniso- and poikilocytosis, moderate hypochromia and microcytosis, and variable polychromasia (Fig. 5). Basophilic stippling was intense in some patients and almost absent in others. Howell-Jolly bodies and malarial parasites were occasionally encountered. Perhaps most surprising was the presence of moderate numbers of spherocytes in the blood films of some patients (Fig. 5). At times, as many as 6 to 8 per cent of the cells showed this change. The appearance of spherocytosis could not be correlated with any data except an increase in the osmotic fragility of a portion of the red cell population. White blood cell counts were often elevated, in part as a result of superimposed infections. Platelet counts were in many instances somewhat low, about half of the patients having less than 300,000 platelets per cubic millimeter. Differential counts frequently revealed a moderate shift to the left and the presence of eosinophilia.

Tests for hemolytic activity supported the hemolytic nature of thalassemia-Hgb E disease. Bone marrow examinations, performed in thirteen patients of this series, showed marked erythrocytic hyperplasia in extremely cellular marrow specimens (Cases 5, 6, 12, 13, 14, 17, 18, 19, 22, 23, 24, 26 and 27; Tables II and III). Hemolytic index determinations were carried out in six

subjects and ranged from 62 in one patient receiving blood transfusions to as high as 508 (normal values up to 20). Van den Bergh determinations were

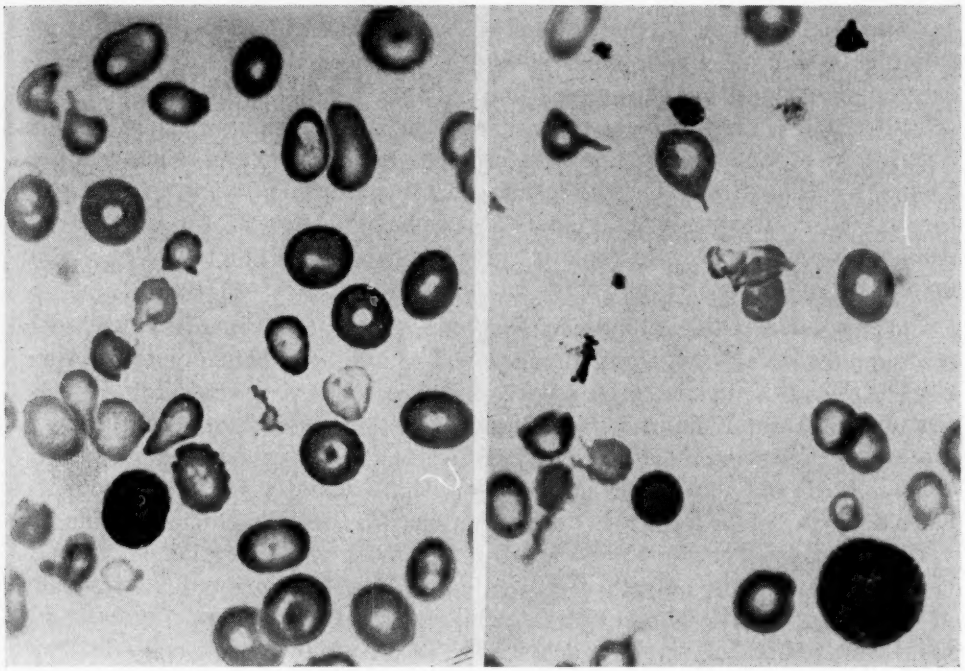


Fig. 5.—Stained peripheral blood films in patients with thalassemia-Hgb E disease. Spherocyte and stippled red cell seen in photomicrograph on right. Nucleated red cell in section at left (×1,200; reduced ⅛).

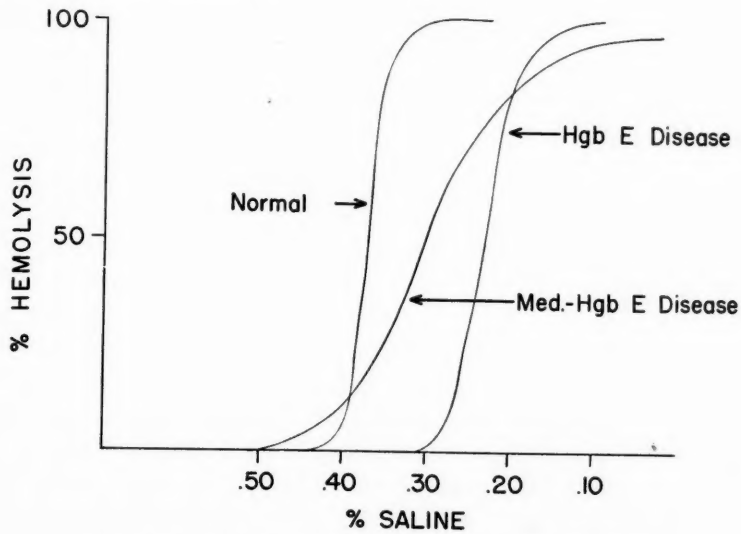


Fig. 6.—Osmotic fragility curves in the Hgb E syndromes.

invariably elevated the indirect reading bilirubin predominating. Red cell survival time studies by the Cr^{51} technique, to be reported separately,[18] were

carried out in eight instances and revealed significantly decreased life spans for the erythrocytes. The Coombs test was questionably positive in one of 12 patients.

Osmotic fragility determinations were performed in almost all cases and resulted in skewed curves which started in the normal range and reached 90 per cent hemolysis in tubes containing between 0.20 and 0.16 per cent saline (Fig. 6). In some instances the red cells began to hemolyze at saline concentrations of 0.46 per cent or greater. The initial stages of hemolysis were frequently difficult to interpret because of the quantity of normal blood given the patients. However, individuals in whom a portion of the erythrocytes demonstrated an increased osmotic fragility were usually noted to have some degree of spherocytosis, but this was not an invariable observation.

Hemoglobin analysis by means of the paper electrophoresis technique showed only Hgb E and Hgb F in the blood of patients who had not been transfused (Fig. 1). In the presence of significant quantities of Hgb F, small amounts of Hgb A may not be detectable by this procedure. There was no indication, however, that Hgb A formed a significant proportion of the total hemoglobin mass except in patients who had recently received exogenous normal erythrocytes. Hgb F determinations by the alkali denaturation test varied from 5.5 per cent to 85.4 per cent. With the exception of those receiving transfusions, only three patients had Hgb F values outside the range of 15 to 40 per cent. These were two children of 21 months and 5 years of age, respectively, both of whom had over 40 per cent Hgb F, and one adult with only 6.0 per cent Hgb F.

Moderately Severe Thalassemia-Hgb E Disease.—Four individuals were felt to be examples of moderately severe thalassemia-Hgb E disease (Cases 29 to 32, Tables II and III). The manifestations of their disease differed somewhat from those observed in the previous group in that they were capable of greater physical effort, required almost no transfusion therapy, and enjoyed moderately good health. Cases 31 and 32 had the onset of menstruation at about age 18, an unusual occurrence in patients with severe thalassemia-Hgb E disease. Furthermore, red cell counts were over 4.0 million per cubic millimeter and fetal hemoglobin levels were generally only slightly increased. In all other respects, however, these patients manifested findings similar to those seen in the more severe group.

Therapeutic Considerations.—No specific form of treatment is available in the thalassemia syndromes. Blood transfusions have been, however, absolutely necessary for the maintenance of life in many patients with Cooley's anemia. From a review of our data, it appears that patients with thalassemia-Hgb E disease have less of a need for blood transfusions than do individuals with Cooley's anemia. These differences may, however, be more apparent than real, since until very recently few facilities for transfusing patients were available in Thailand. Nevertheless, it is our impression that even had blood been readily available, individuals with thalassemia-Hgb E disease would have required fewer transfusions than a similar group of patients with Cooley's

anemia. On the other hand, a number of the children with extremely severe disease required as many transfusions as the most distressing examples of Cooley's anemia.

During the course of the original study on thalassemia in Thailand by Minnich and associates,[1] it was noted that splenectomy appeared to have benefited a number of the patients in that growth and development progressed somewhat and the general health of the individuals improved. Furthermore, the transfusion requirement seemed to be definitely reduced. Little other change in the status of the hemolytic anemia, however, was apparent. A similar impression was unavoidable when the histories from the present group of cases were reviewed in which three individuals had been splenectomized from four months to five years before study. None has required blood transfusions since surgery, two seem to have developed some improvement in their red cell and hemoglobin levels, and the youngest individual appears to be growing more rapidly than before the operation. As a consequence, we have subjected twelve more patients to splenectomy, in an effort to evaluate this procedure. It is still too early to determine the outcome of this approach, but follow-up studies will be reported at a later date when adequate data are available.

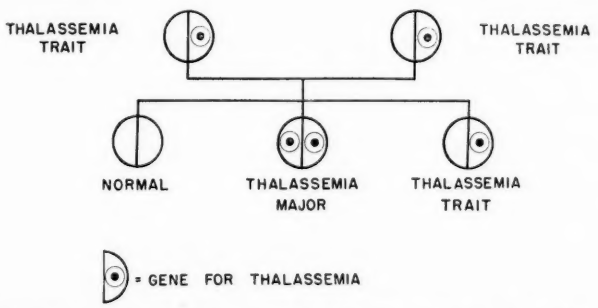


Fig. 7.—Genetic representation of thalassemia major and minor (after Valentine and Neel).

Genetic Considerations.—According to the theory of Valentine and Neel,[19, 20] thalassemia major represents the homozygous condition for whatever gene or genes are affected in the thalassemia syndromes. The thalassemia trait is, on the other hand, thought of as the heterozygous form of the same defect (Fig. 7). Clinical experience indicates a wide spectrum of syndromes of varying degrees of severity, ranging from classical Cooley's anemia to the mildest forms of thalassemia minor.[16, 21] It seems unlikely, therefore, that a single set of allelomorphs is responsible for this disorder. However, for the sake of discussion, it is expedient to employ the simple concept of heterozygosity or homozygosity of a single set of genes as representing the defect in thalassemia, keeping in mind that in all probability this assumption is not entirely correct.

The nature of the genetic defect in the thalassemia syndromes is not at all understood. It has been postulated that in thalassemia an inability to incorporate iron into the normal hemoglobin molecule exists or that there is a defect in the formation of normal adult hemoglobin itself.[7, 14] Studies by a number of investigators have shown a rather consistent elevation of fetal

hemoglobin in the major thalassemia syndromes.[11, 22-24] It seems very likely that one effect of the thalassemia gene is to enhance the formation of Hgb F, either directly or by a suppressive action on Hgb A formation. It is also possible that the thalassemia gene acts at the same locus as Hgb F, although no experimental data exist relative to this postulate.[14]

Evidence accumulated from extensive studies of thalassemia-Hgb S disease, particularly by Silvestroni and Bianco,[2, 25, and others] suggests that the gene or genes for thalassemia are located on chromosomes different from those determining the abnormal hemoglobins. Usually thalassemia-Hgb S disease results when one parent is heterozygous for Hgb S and the other parent heterozygous for thalassemia. Since the two abnormal genes involved are apparently on separate chromosomes, it is theoretically possible for an individual with thalassemia-Hgb S disease to have an offspring with sickle cell trait, thalassemia trait, or thalassemia-Hgb S disease or for the child to be normal. Furthermore, it is theoretically possible for an individual to be homozygous or heterozygous for the thalassemia gene and at the same time to be homozygous or heterozygous for an abnormal hemoglobin. One can only speculate whether homozygosity for both these defects would result in a lethal combination or in what manner homozygosity for one and heterozygosity for the other might become manifest. The variations in symptomatology that could arise under such circumstances may well explain some of the gradations encountered clinically in the thalassemia syndromes.

The gene for Hgb E seems to be an allelomorph of the genes for Hgb A, C, or S. One would expect, therefore, a genetic pattern in thalassemia-Hgb E disease similar to that seen in the corresponding combination involving Hgb S and thalassemia. Usually, one parent will be a carrier for Hgb E, the other for thalassemia (Fig. 8). If each parent is heterozygous for his respective genetic defect, one-fourth of the children would be expected to harbor the combination referred to as thalassemia-Hgb E disease. Furthermore, a single individual with thalassemia-Hgb E disease married to a normal person may have children who are normal, are Hgb E trait or thalassemia trait carriers, or who suffer from thalassemia-Hgb E disease (Fig. 9). Matings involving homozygotes for Hgb E with individuals harboring the genetic defect for thalassemia with or without an abnormal hemoglobin will alter the expected ratios and may give rise to a variety of hematologic syndromes that deviate from the usual clinical manifestations seen in thalassemia-Hgb E disease.

The 32 patients with thalassemia-Hgb E disease described in the above sections represented 28 different kinships. In addition to the 32 patients, five more siblings were known to be afflicted with the same disorder. These 28 family groups produced a total of 121 viable offspring, of whom 56 are living and well, although an indeterminate number have the Hgb E trait or the thalassemia trait. The remaining 27 children are dead and, on historical grounds, at least 12 of them succumbed to thalassemia-Hgb E disease. Thus, 49 of the 121 children (40 per cent) probably represent instances of this severe hemolytic anemia. Although this percentage is higher than the expected ratio of one in four, three factors undoubtedly have influenced the results.

First, many pregnancies did not proceed to term; second, the diagnosis is only inferred in some of the cases; and third, not all parents were available for study of their hemoglobin types. Consequently, the number of parents

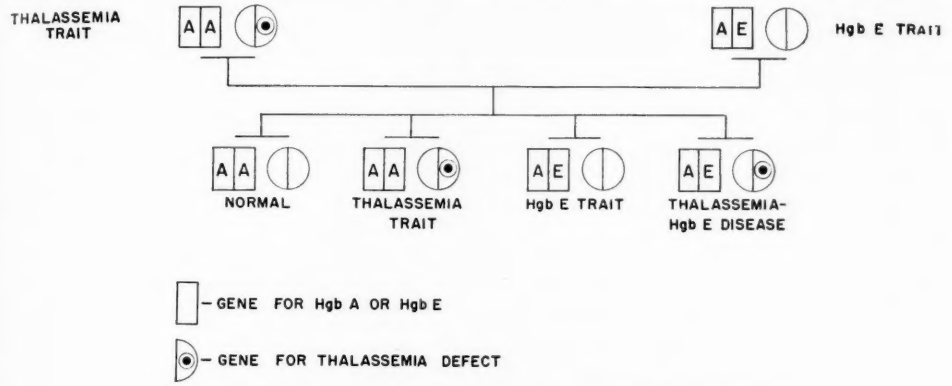
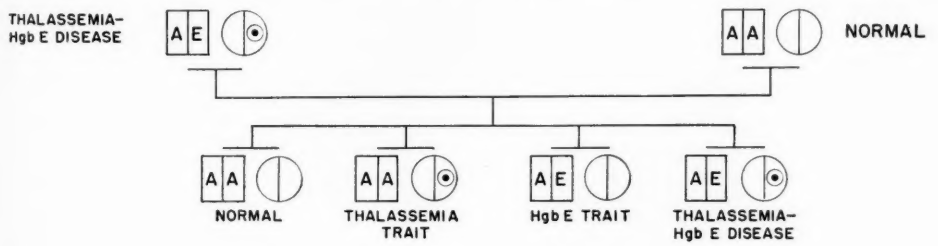


Fig. 8.—Genetic representation of thalassemia-Hgb E disease.

homozygous for Hgb E or thalassemia could not be determined. Homozygosity for the abnormal pigment or the presence of thalassemia-Hgb E disease in a parent would weight the results in the direction noted. It would seem, however,



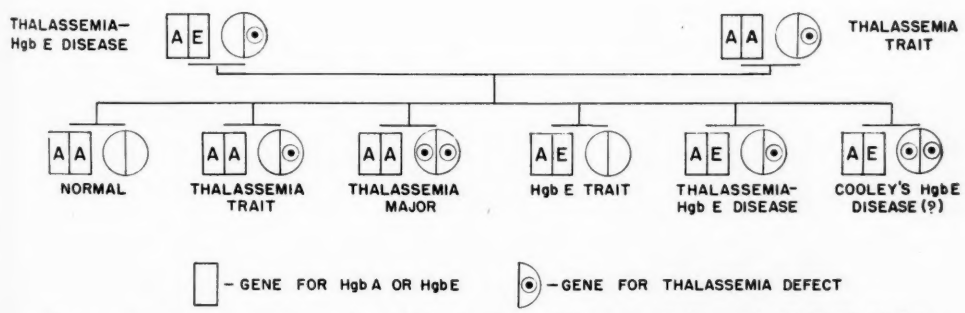


Fig. 9.—Genetic represention of possible matings leading to thalassemia-Hgb E disease.

that the observed incidence of thalassemia-Hgb E disease does not differ significantly from the expected incidence, assuming two independent chromosomes are involved in the pathogenesis of this syndrome.

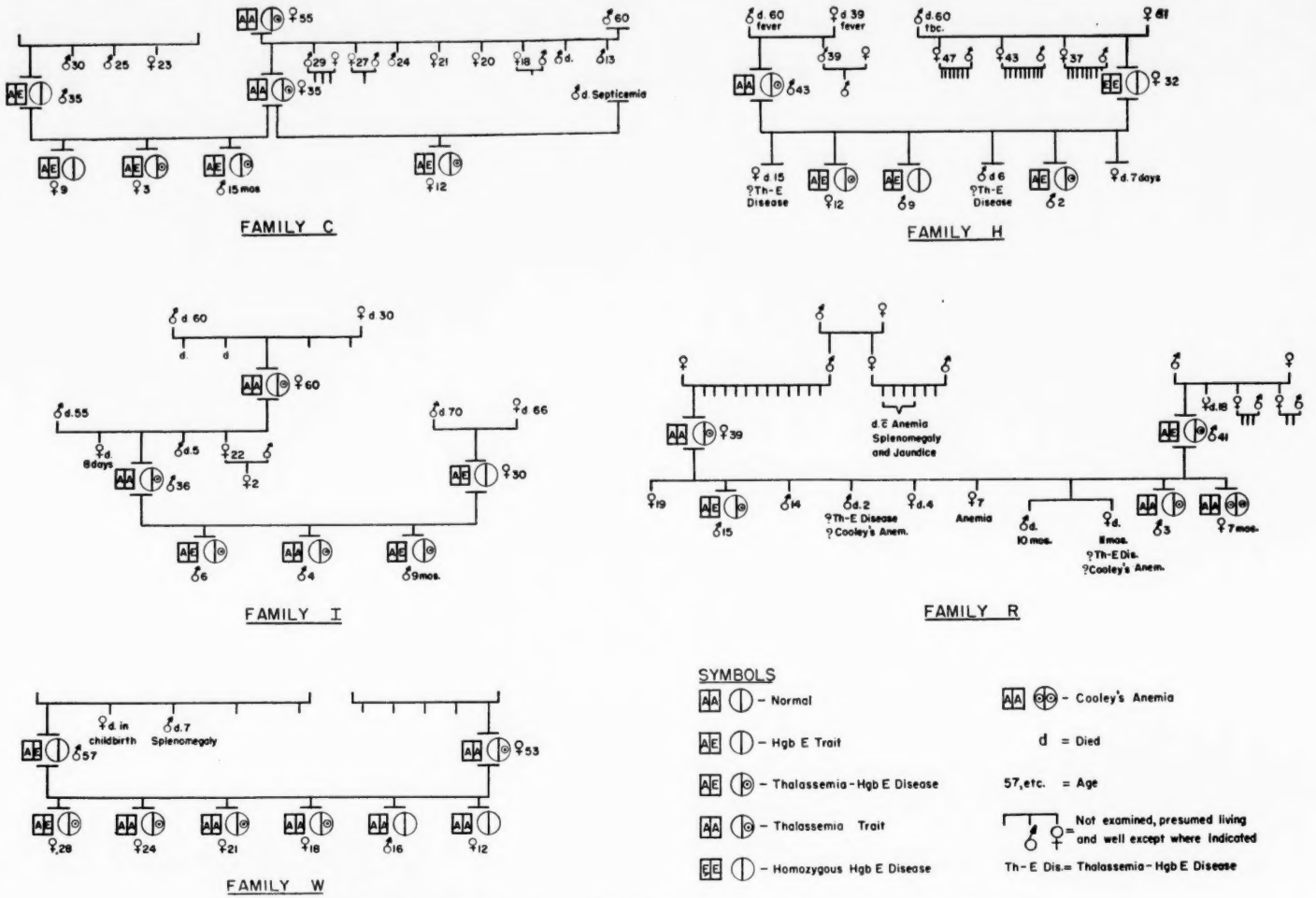


Fig. 10.—Kinships of five representative families with thalassemia-Hgb E disease.

No family studies were available in seven of the 28 kinships. In eighteen families both parents were studied, and in every case Hgb E was present in the red cells of one and absent in the erythrocytes of the other. At least one of the kinships involved a mother probably homozygous for Hgb E, another a father with thalassemia-Hgb E disease. In the remaining three families, only one parent was investigated. Hgb E was detected in two and hematologic data compatible with thalassemia minor were noted in one.

Hematologic Data in the Nonhemoglobin E Parent.—The lack of critical criteria for the diagnosis of the mildest forms of thalassemia makes this diagnosis a difficult one of which to be certain.[7, 28, 29, 30] The presence of target cells, alone or in combination with a one tube osmotic fragility test, has been used as a screening procedure to determine the prevalence of the thalassemia trait[31] but the nonspecific nature of this morphologic abnormality, especially in a population with a high incidence of parasitic and hepatic disease, renders the finding unsuitable for evaluation. Other criteria generally employed for reaching a diagnosis of the milder forms of thalassemia are the low MCV and MCHC, hypochromic polycythemia, and basophilic stippling.[29, 32] On the other hand, it has been pointed out that determination of the MCHC frequently results in values near the normal range and that hypochromia, as such, is not a necessary adjunct in this syndrome.[32, 33] A definite decrease in the osmotic fragility of the erythrocytes is usually deemed necessary for identifying the thalassemia minor syndrome.[28-30] Finally, not infrequently, the diagnosis of thalassemia minor in a parent can be arrived at only by inference based on the fact that a typical case of Cooley's anemia exists in a given kinship.

In view of these difficulties, it seemed of some interest to study the parents of our cases of thalassemia-Hgb E disease who did not carry the gene for Hgb E. Eighteen parents and six other relatives were available for hematologic analysis in the 28 kinships under investigation. Although the data are compatible with a diagnosis of thalassemia minor they are frequently not strikingly abnormal and could well represent other genetic defects. In the present state of our knowledge, however, it seems best to classify these individuals as carriers of the gene for thalassemia, while keeping in mind the difficulties inherent in arriving at that diagnosis.

The most significant hematologic data are summarized in Table IV. Red cell counts as high as 7.35 million per cubic millimeter were seen. Hemoglobin levels varied from 8.8 to 15.1 Gm. per 100 ml. In only three subjects was the MCV 79 c.μ or over. Perhaps the chief atypical feature was the observation that only seven (only three of the parents) individuals had an MCHC of less than 31 per cent. Target cell counts ranged from 0.1 to 19.1 per cent in the parents and up to 37.3 per cent among the siblings. However, only one of the parents had over 10 per cent target cells. The osmotic fragility curve approximated normal in only one of the individuals studied, while most subjects showed 90 per cent hemolysis of their erythrocytes at saline concentrations of 0.28 per cent or less. Basophilic stippling was infrequent but not diligently searched for. Anisocytosis and poikilocytosis were not prominent. Finally,

Fig. 10.—Kinships of five representative families with thalassemia-Hgb E disease.

TABLE IV. LABORATORY DATA IN INDIVIDUALS WITH THALASSEMIA MINOR

CASE NO.	MEMBER FAMILY GROUP	RELATION-SHIP	AGE	SEX	RBC	Hgb	PCV	MCV	MCHC	RETICS	TARGET CELLS	OSMOTIC FRAGILITY	I.I.	vdB	Hgb F	SPLENO-MEGALY	HEPATO-MEGALY
1	B	Mother	36	F	5.22	14.1	36.5	70	31	0.4	1.2	0.38-0.28	7.5	$\frac{0.25}{0.35}$	N	-	-
2	C	Mother	35	F	4.67	9.9	32.5	70	31	0.2	-	0.39-0.26	5	$\frac{0.05}{0.20}$	N	0.0	CM
3	D	Father	-	M	4.93	14.7	44	89	34	0.8	5.2	0.38-0.24	5	$\frac{0.32}{0.77}$	N	-	-
4	E	Father	-	M	5.57	14.7	44	79	33	0.2	0.1	0.40-0.28	5	$\frac{0.47}{0.64}$	N	-	-
5	F	Father	-	M	6.16	15.1	46	74	33	0.8	7.1	0.42-0.30	10	$\frac{0.22}{0.55}$	N	-	-
6	G	Father	-	M	5.71	12.8	40	71	32	0.2	3.8	0.42-0.24	5	$\frac{0.0}{0.62}$	N	-	-
7	H	Father	43	M	5.18	13.2	40	77	33	0.4	-	0.34-0.18	5	-	N	CM	0.0
8	I	Father	36	M	7.35	13.8	46	63	30	1.0	2.5	0.37-0.24	5	-	N	0.0	CM
9	I	Paternal Grandmother	60	F	5.13	11.0	35	68	31	0.0	8.0	0.36-0.16	5	-	N	0.0	3.0
10	I	Brother	4	M	5.26	10.4	32	61	33	0.2	35.0	0.33-0.21	5	-	N	0.0	CM
11	J	Mother	37	F	6.2	12.4	38.5	62	32	0.4	3.8	0.37-0.22	7.5	$\frac{0.13}{0.97}$	N	0.0	0.0
12	K	Father	-	M	5.98	14.3	42	70	34	0.4	1.1	0.38-0.28	5	$\frac{0.0}{0.47}$	N	-	-
13	L	Father	-	M	5.32	11.6	36	64	32	0.6	4.8	0.38-0.28	5	-	N	-	-

14	N	Mother	–	F	5.14	12.1	37.5	73	32	0.8	3.8	0.38-0.24	7.5	0.30 / 0.73	N	–	–
15	O	Mother	30	F	4.61	10.4	32	69	33	0.4	8.8	0.38-0.24	5.0	0.00 / 0.61	4.1	–	–
16	P	Father	48	M	5.58	11.3	35	63	32	0.6	–	0.35-0.21	5	–	2.1	0.0	2.0
17	R	Mother	39	F	5.23	11.4	39	75	29	0.6	9.6	0.38-0.24	10	0.41 / 0.61	9.1	0.0	0.0
18	R	Brother	3	M	5.30	10.6	38	74	28	0.4	6.6	0.38-0.16	5	0.19 / 0.31	N	0.0	0.0
19	S	Father	68	M	5.65	13.5	42	74	32	2.2	19.1	0.34-0.28	5	0.13 / 0.34	N	0.0	CM
20	W	Mother	53	F	4.29	10.4	34	79	31	–	6.8	0.42-0.28	5	–	N	0.0	1.5
21	W	Sister	18	F	5.05	10.7	37	73	29	–	3.2	0.38-0.26	10	0.18 / 1.06	N	0.0	0.0
22	W	Sister	24	F	5.28	10.7	37	70	29	–	9.9	0.36-0.28	7.5	0.14 / 0.38	3.1	0.0	0.0
23	W	Sister	21	F	5.54	11.3	38	69	27	–	37.3	0.38-0.27	5	0.18 / 0.72	N	0.0	0.0
24	Z	Mother	–	F	4.0	8.8	27.5	69	32	0.6	2.0	0.38-0.28	7.5	0.25 / 0.50	N	–	–

See Legend under Table I.

hemoglobin analysis indicated the presence of only Hgb A on paper electrophoresis, while the alkali denaturation test showed a normal quantity of Hgb F in all but five individuals. The latter had up to 9.1 per cent Hgb F, an observation occasionally made in patients with the thalassemia syndromes.[11, 24]

Representative family studies are shown in Fig. 10, and a short summary of several of these kinships is presented below.

CASE REPORTS

FAMILY C (Fig. 11).—The propositi of this kinship consisted of Cases 4, 5, and 6 (Table II). The father (Case 16, Table I) has the Hgb E trait. The mother of the

Fig. 11.—Family C from left to right: Case 4, Table II, Case 34, Table I, Case 6 and Case 5, Table II. Their ages are 1¼, 9, 12, and 3 years, respectively.

propositi (Case 2, Table IV) is an example of the thalassemia trait. Three children resulted from this marriage; two with thalassemia-Hgb E disease (Cases 4 and 5, Table II), and one with the Hgb E trait (Case 34, Table I). One child was the offspring of this woman by a former marriage and is an example of severe thalassemia-Hgb E disease (Case 6, Table II). Although the father of this child is dead and none of his relatives could be studied for the presence of Hgb E there is every likelihood that he too carried the gene for Hgb E. The maternal grandmother was felt to have the thalassemia trait.

FAMILY H.—The propositus was Case 12 (Table II) with thalassemia-Hgb E disease. The father (Case 7, Table IV), a storekeeper living some 20 kilometers east of Bangkok, shows manifestations compatible with thalassemia minor. The mother of the propositus

(Case 3, Table V) is believed to have homozygous Hgb E disease. Three of the six offspring born to this couple were available for study. Two have thalassemia-Hgb E disease (Cases 11 and 12, Table II) and one has Hgb E trait (Case 20, Table I). The other three children are dead. The eldest daughter developed pallor, jaundice, and an enlargement of the abdomen at age three and died at age 13. The father's concern about the abdominal enlargement led him to perform an autopsy on his own child on the kitchen table; he noted a large organ filling the left half of the abdomen extending into the iliac fossa. The second dead offspring developed similar symptoms at age 1 and died at age 5. The father again examined the abdominal contents and found massive splenomegaly. A third child died at 7 days of age of unknown causes and was not examined by the father.

FAMILY I.—The propositus (Case 13, Table II) has severe thalassemia-Hgb E disease. His mother (Case 35, Table I) is an example of the Hgb E trait. His father and paternal grandmother (Cases 8 and 9, Table IV, respectively) are examples of thalassemia minor. In addition to the propositus, two other children resulted from this marriage. One, a nine-month-old male, not included in this study, has typical thalassemia-Hgb E disease. The other, a boy 4 years of age, is considered to be a thalassemia trait and is listed as Case 10, Table IV.

FAMILY R.—The propositus, Case 22, Table II, has severe thalassemia-Hgb E disease. The patient's father, with atypical thalassemia-Hgb E disease, is Case 3, Family KK, Table VI. the mother of the propositus (Case 18, Table IV) is one of 13 children, all of whom were thought to be healthy. She is, however, an example of an intermediate form of thalassemia. Her paternal aunt gave birth to six children, four of whom died with anemia, splenomegaly, and jaundice and are probable examples of thalassemia-Hgb E disease or Cooley's anemia. The father of the propositus (Case 3, Table VI) had four siblings. When first seen, the father was believed to have homozygous Hgb E disease, but family studies on his children revealed several without any sign of the abnormal pigment. He must be classified, therefore, as a very atypical instance of thalassemia-Hgb E disease (vide infra). There were ten children in the family, four of whom died with a syndrome resembling thalassemia-Hgb E disease or Cooley's anemia. Of the remaining six, the propositus (Case 22, Table II) is an example of thalassemia-Hgb E disease. A sister and a brother, aged 19 and 14, respectively, are said to be well but were not studied. Another sister, aged seven, is reported to have had signs of anemia at age 2. The second youngest member of this kinship is a 3-year-old boy (Case 18, Table IV) with no evidence of Hgb E but who has hematologic data suggestive of thalassemia. The youngest sibling, a 7-month-old girl, was admitted to the hospital in a moribund state with weakness, pallor, and jaundice of about five months' duration. A mass in the left side of the abdomen became apparent four months before admission. Physical examination revealed the liver down about 3 cm. and the spleen enlarged to 4.5 cm. below the left costal margin. Her red cell count was 1.56 million per cubic millimeter, hemoglobin 3.2 Gm. per 100 ml., hematocrit 11 per cent, MCV 70 c.μ, MCHC 29 per cent, and reticulocytes 4.8 per cent. The differential revealed a marked shift to the left with 16 NRBC per 100 white blood cells. Anisocytosis, poikilocytosis, and hypochromia were marked. Paper electrophoresis revealed Hgb A and Hgb F but no Hgb E. Fetal hemoglobin determination by the alkali denaturation technique showed 66.7 per cent Hgb F. This girl, therefore, represents an instance of Cooley's anemia. Although insufficient data pertaining to the blood groups of this kinship are available, there is no evidence to suggest impaternity. It must be assumed, therefore, that the family represents a complex genetic picture in which both thalassemia minor and thalassemia-Hgb E disease are harbored. Of the six genetic syndromes possible in such a mating, at least three are certainly present in this kinship (thalassemia minor and major and thalassemia-Hgb E disease).

FAMILY W.—The propositus (Case 27, Table II) has thalassemia-Hgb E disease. The father (Case 27, Table I) has Hgb E trait. His oldest sister died at delivery, and a brother died at the age of 7 with a large mass in the left upper quadrant. The patient's

TABLE V. CLINICAL AND LABORATORY DATA IN PATIENTS WITH PROBABLE HOMOZYGOUS HGB E DISEASE

CASE NO.	MEMBER FAMILY GROUP	AGE	SEX	RACE		NO. OF SIBLINGS		CHILDREN	HISTORY OF					SPLENOMEGALY	HEPATOMEGALY	ICTERUS	REMARKS
				THAI	OTHER	LIVING	DEAD		ANEMIA	FEVER	ABDOMINAL PAIN	JOINT PAINS	WEAKNESS				
1	CC	2⅔	F	4/4	–	1	0	–	0	±	0	–	0	0	0	0	Upper respiratory infection. Ascariasis
2	DD	16	F	4/4	–	2	2	–	0	0	0	0	0	0	0	0	Influenza
3	EE H	32	F	4/4	–	3	0	6	0	0	0	+	+	0	0	±	Mother of family H
4	FF	35	M	–	–	–	–	–	0	0	0	0	0	0	0	0	Father of patient with Hgb E trait
5	GG	37	M	4/4	–	6	2	–	+	0	0	++	++	2.0	0	+	Bleeding peptic ulcer
6	HH	40	F	–	–	–	–	–	–	–	–	–	–	–	–	–	Mother of patient with thalassemia-Hgb E disease

CASE NO.	RBC	Hb.	PCV	MCV	MCH	MCHC	WBC	RETICS	PLATELETS	NRBC	TARGET CELLS	ANISOCYTOSIS	POIKILOCYTOSIS	HYPOCHROMIA	MICROCYTOSIS	VdB	OSMOTIC FRAGILITY	Hgb F	H.I.	BONE MARROW E:M
1	3.97	7.9	26	66	20	30	10.1	0.4	–	0	41.2	2+	2+	2+	+	0.14/0.44	0.30-0.18	6.4	–	
2	4.02	8.8	28	69	22	31	3.8	1.0	960	–	–	–	–	–	–	0.28/1.17	0.34-0.20	3.1	–	
3	4.82	11.1	33	69	23	34	13.8	1.4	440	0	48.0	+	0	+	+	–	0.30-0.19	2.4	–	1:3
4	5.16	11.7	37	72	23	29	–	–	–	0	25.4	+	0	+	0	0.30/0.73	0.36-0.24	1.2	–	
5	5.55	13.2	39.5	71	24	34	–	1.4	850	0	25.0	–	–	–	–	0.19/1.13	0.32-0.19	6.0	11	1:1
6	5.2	10.1	34	65	21	32	7.8	1.4	Many	0	11.2	+	+	++	+	–	–	3.7	–	–

See Legend under Table I.

mother has thalassemia minor and is Case 20 (Table IV). Six children were born in this family, of whom the patient is the oldest. The other five consist of three with thalassemia minor (Cases 21, 22, and 23, Table IV), and two healthy children, aged 16 and 12, with completely normal hematologic findings.

HOMOZYGOUS HEMOGLOBIN E DISEASE

Six patients were observed with a clinical and hematologic syndrome remarkable not only in its uniform pattern but also in its striking dissimilarity from thalassemia-Hgb E disease (Table V). These six patients are believed to be homozygous for Hgb E and their disease may consequently be referred to as homozygous or "pure" Hgb E disease. Unfortunately, in no case was it possible to study both parents and thus demonstrate the necessary genetic criteria for homozygosity. One kinship, Family H, however, consisted of a mother, who has what appears to be homozygous Hgb E disease, and her six children, five of whom probably had the gene for Hgb E (Fig. 10). A second family, under investigation by Na-Nakorn and Minnich[34] and not included in this report, fulfills all genetic criteria for homozygosity for Hgb E and presents remarkably similar clinical and hematologic manifestations. Because of these considerations, the syndrome described below is felt to represent homozygous Hgb E disease.

Although incomplete historical and laboratory data are available on the six individuals with what is believed to be homozygous Hgb E disease, sufficient information is at hand to describe this syndrome briefly. The patients ranged in age from two and two-thirds to 40 years. Three of the subjects were married and had a total of eight children. All the patients were in fair to good health, although a history of tiredness, easy fatigability, and mild arthralgias could usually be elicited.

Physical examinations, carried out in five of the six patients, were relatively unremarkable except for the somewhat small stature of the subjects. Slight icterus appeared to be present, but van den Bergh determinations usually failed to support this observation. The spleen was palpable 2 cm. below the left costal margin in one; the liver 1 cm. down in a second. Other abnormalities related to their hematologic status were absent.

Hematologic studies indicated the presence of a microcytic normochromic anemia, with the erythrocyte counts usually falling within the normal range. MCV determinations were between 65 and 72 c.μ, while MCHC values ranged from 29 to 34 per cent. The lower mean corpuscular hemoglobin concentrations appeared in individuals who had reason for an iron deficiency state unrelated to their basic hematologic disease. Reticulocyte counts were consistently normal, and nucleated red cells were not seen in the peripheral blood. The red cell morphology was only mildly deranged, except for a striking increase in the percentage of target cells, ranging from 25 to as many as 75 per cent on some blood films (Fig. 2). Osmotic fragility studies demonstrated a marked increase in the resistance of the erythrocytes to hypotonic saline, the fragility curve being shifted fairly symmetrically to the right in contrast to the skewed curve usually seen in thalassemia-Hgb E disease (Fig. 6). Bone

TABLE VI. DATA IN ATYPICAL CASES OF THE HGB E SYNDROMES

CASE NO.	MEMBER FAMILY GROUP	AGE	SEX	RACE		NO. OF SIBLINGS		CHILDREN	DURATION OF SYMPTOMS OF		SPLENOMEGALY	HEPATOMEGALY	ICTERUS	X-RAYS	TRANSFUSIONS	REMARKS
				THAI	OTHER	LIVING	DEAD		ANEMIA	SPLENOMEGALY						
1	II	32	F	4/4	-	4	2	4	3 mos.	0	0	1.5	0	Normal	-	Cervical adenitis
2	JJ	35	F	4/4	-	-	-	10	4 mos.	10 yrs.	3	16	++	Normal	None	
3	KK	41	M	4/4	-	2	1	10	0	4 yrs.	0	0	+	Normal	None	Father–family R
4	LL	19	F	4/4	-	4	0	0	3 mos.	3 mos.	6.5	5.0	++++	?Compatible with Cooley's	None	Cholelithiasis ?Malaria

	RBC	Hgb	PCV	MCV	MCH	MCHC	WBC	RETICS	PLAT.	NRBC	TARGET CELLS	ANISOCYTOSIS	POIKILOCYTOSIS	HYPOCHROMIA	MICROCYTOSIS	STIPPLING	VdB	OSMOTIC FRAGILITY	Hgb F	H.I.	BONE MARROW E:M
1	3.46	8.6	32	93	24	27	-	0.9	Many	0	64.6	2+	2+	0	0	0	0.01/0.22	0.32-0.18	17.7	-	1:1
2	3.97	8.7	31	78	22	28	-	0.5	Many	6	>20.0	-	-	-	-	-	0.38/2.80	0.44-0.16	42.0	-	1:2
3	5.88	11.7	38	65	20	31	14.2	0.6	480	0	78.6	-	-	-	-	-	0.09/0.83	0.30-0.13	3.0	16.8	1:1
4	3.6	7.3	25	70	20	29	6.4	0.8	360	1	20.0	3+	2+	0	2+	+	0.64/8.35	0.40-0.16	1.06	-	-

See Legend under Table 1.

marrow examinations, done in two of the patients, showed a normal cellular pattern in one and minimal erythrocytic hyperplasia in the second. The hemolytic index determination in the latter was 16, well within normal limits. No bone changes were demonstrable in the one patient studied roentgenographically. Although no evidence exists at present to support the concept of increased hemolysis in homozygous Hgb E disease, further study may uncover a mild hemolytic mechanism.

Hemoglobin analysis revealed only Hgb E on paper electrophoresis, although up to 6.4 per cent Hgb F could be detected by the alkali denaturation technique (Fig. 1).

Genetic studies on the patients with homozygous Hgb E are incomplete. By history alone, no sibling of an individual in this group, however, was felt to have had an anemia or other hematologic disease. This observation is in marked contrast to the experience with family studies in the thalassemia-Hgb E disease group, where approximately one-third of the siblings had similar complaints and findings. Perhaps the kinship which best supports the idea of homozygosity for Hgb E is Family H, described in the previous section, where each of the three living children had Hgb E, while two of the dead offspring in all probability succumbed to thalassemia-Hgb E disease (Fig. 10).

Atypical Forms of Thalassemia-Hemoglobin E Disease and Homozygous Hemoglobin E Disease.—Four patients were observed with hematologic syndromes believed to be either thalassemia-Hgb E disease or homozygous Hgb E disease but characterized by most unusual features, which made it difficult to classify these cases with certainty. The pertinent data relating to these patients are included in Table VI and detailed summaries of Patients K. K. and L. L. follow.

CASE REPORTS

PATIENT I. I.—The patient was considered to be atypical, although her hemoglobin pattern is that of thalassemia-Hgb E disease, because the increase in target cells plus the absence of signs of hemolysis, bone changes, and physical abnormalities suggest more the manifestations of homozygous Hgb E disease.

PATIENT J. J.—This patient also had findings suggestive of thalassemia-Hgb E disease, but the anemia was only mild and reticulocytosis was absent. Although incomplete laboratory data make it difficult to evaluate this patient completely, the changes strongly suggest a very mild form of thalassemia-Hgb E disease.

PATIENT K. K.—This patient, a 41-year-old man, the father of one of the patients with thalassemia-Hgb E disease (Case 22, Family R, Tables II and III), presents the most unusual picture of all. When originally seen, he was felt to represent a typical example of homozygous Hgb E disease, but family studies make this diagnosis untenable. Except for an episode of splenomegaly and jaundice four years prior to examination, no other relevant history was obtained. Physical examination revealed a healthy-looking man of small stature who had minimal icterus and a slightly enlarged spleen. The hematologic observations were indistinguishable from the findings in homozygous Hgb E disease. Genetic studies, however, suggest that he cannot be homozygous for Hgb E because of the absence of Hgb E in two of his children. The details of this kinship are summarized in the previous section (Fig. 10). It must be assumed, therefore, barring impaternity or technical error, that the father is heterozygous for Hgb E and that he represents a very mild and unusual form of thalassemia-Hgb E disease.

PATIENT L. L.—The final patient, a Thai girl, 19 years of age, is believed to be either an example of homozygous Hgb E disease or of thalassemia-Hgb E disease complicated by superimposed cholecystitis, cholelithiasis, liver disease, and possibly malaria. When admitted to the hospital, she complained of right upper quadrant pain, jaundice, pruritus, and dark urine for three months. Signs of anemia were present for only one week. Physical examination revealed a markedly icteric woman with some pallor but no obvious evidence of chronic disease. The liver was palpable 5.0 cm. and the spleen 6.5 cm. below the costal margins. The hematologic values are included in Table VI and show a microcytic hypochromic anemia without reticulocytosis. Target cells were increased and 1 nucleated red blood cell per 100 WBC was seen. The red cells showed marked anisocytosis, moderate poikilocytosis and microcytosis, and stippling which resembled Schüffner's granules. Icterus index and van den Bergh determinations support the diagnosis of obstructive jaundice. Roentgenograms of the bones were somewhat abnormal, but a definitive diagnosis of hemolytic anemia could not be made from these studies. Electrophoresis revealed only Hgb E, while the alkali denaturation test showed a normal value for Hgb F. Although the majority of the features of this case fit in well with the findings of homozygous Hgb E disease, several aspects are unusual. Particularly difficult to ascribe to pure Hgb E disease are the hypochromia, abnormalities of red cell size and shape, and the hepatosplenomegaly. Whether the complicating diseases are responsible for these atypical features will have to be determined by re-examination at a later date.

DISCUSSION

Most investigators now accept the concept that the human hemoglobins, with the exception of Hgb F, are controlled by a single set of genetic factors and that the gene for any of the abnormal pigments may replace one or both genes for Hgb A.[14, 33, 35, 36] The condition in which one gene for an abnormal hemoglobin replaces a gene for Hgb A is commonly referred to as a heterozygous hemoglobin trait. When both genes for Hgb A are replaced by those of the abnormal compound, a homozygous or pure hemoglobin disease results. Other combinations, involving the mixed heterozygous state for two abnormal hemoglobins and the combination of an abnormal pigment with the genetic defect for thalassemia or hereditary spherocytosis may give rise to a complex variety of hematologic syndromes.[14] The family studies presented in this communication are entirely consistent with the concept that Hgb E, like Hgb S, C, or D, is an allele of Hgb A. Hgb E has, furthermore, been detected in the heterozygous and homozygous forms, as well as in association with the gene for thalassemia.

Hgb F, unlike the other hemoglobin types, seems to be under independent genetic control.[14, 27, 33, 37] Data obtained in children suggest that as the individual ages, the expressivity of the gene for Hgb F decreases so that within a few years after birth very little of the embryonic pigment is produced.[14, 38] The mechanism for the suppression of Hgb F formation is not known, but it has been postulated that Hgb A and its alleles tend to inhibit in varying degrees the formation of the fetal compound.[14, 27, 37] On the other hand, certain factors are believed to enhance the expressivity of Hgb F, and among these thalassemia is most active. In the Hgb E trait, no abnormal quantity of Hgb F is detected, presumably due to the strong suppressive action of the gene for Hgb A. Homozygous Hgb E disease is characterized by a small increase in the percentage of Hgb F, since Hgb E is felt to be a less potent inhibitor of Hgb F.

In the presence of the strong enhancing ability of the thalassemia gene, 15 to 40 per cent of the hemoglobin is of the fetal variety in thalassemia-Hgb E disease.

Studies by Silvestroni and Bianco and others, already referred to, suggest that the hereditary factors controlling the abnormal hemoglobins and the thalassemia characteristics are located on different chromosomes.[2, 14, 25, 26, 27] Individuals with thalassemia-Hgb E disease would presumably be heterozygous for both Hgb E and thalassemia (Fig. 8). It is of some interest, therefore, to note that Hgb A was rarely if ever detected in electrophoretic studies on blood specimens from patients with thalassemia-Hgb E disease who had not been transfused. In thalassemia-Hgb S disease, the usual hemoglobin formula is Hgb S + Hgb A + Hgb F with percentages varying from 65 to 80 per cent Hgb S, 2 to 20 per cent Hgb A, and 5 to 20 per cent Hgb F.[3, 14, 27, 39] One case of thalassemia-Hgb S disease, reported by Humble and co-workers,[40] had almost 100 per cent Hgb S, with little if any Hgb A and Hgb F. It is difficult to explain the preponderance of the abnormal hemoglobin, as well as the virtual absence of Hgb A, in the light of the presumed heterozygous state of the pathologic hemoglobin. Several factors, however, may contribute to this observation. In other syndromes involving the abnormal hemoglobins in the heterozygous state, there is rarely an equal distribution of the two pigments. Thus, in sickle cell trait, as well as in all other hemoglobin traits, Hgb A forms from 55 to 70 per cent of the total hemoglobin mass, despite the presence of only one gene for Hgb A.[41, 42] Consequently, unknown factors regulating the expressivity of the genes for the hemoglobins must be operating even in this most elementary combination. Furthermore, in uncomplicated thalassemia major, Hgb F is present in amounts of up to 90 per cent, in spite of the presence of Hgb A, presumably in the homozygous form.[11, 24] Whether the gene for thalassemia acts at the locus of the gene for Hgb F and thus directly enhances its production or whether the thalassemia factor suppresses the formation of Hgb A are problems already referred to. It may be postulated, however, that when thalassemia is present, even in the heterozygous form, Hgb A production is suppressed, while Hgb F production is enhanced. In addition, the gene for thalassemia seems to increase the percentage of any associated abnormal pigment. Coupled with the gene for an abnormal hemoglobin, the thalassemia factor may then increase the expressivity of both the abnormal pigment and Hgb F to such an extent that little if any Hgb A is formed. Thus, the ratios of hemoglobin concentrations found in thalassemia-Hgb S disease and thalassemia-Hgb E disease may be explained.

Other factors which could be involved in the absence of Hgb A in thalassemia-Hgb E disease seem to be of lesser importance. None of the patients reported were felt to be homozygous for Hgb E and heterozygous for thalassemia, although this combination presumably could give rise to almost 100 per cent Hgb E and no Hgb A. Homozygosity for thalassemia and heterozygosity for Hgb E, also believed to be absent in the patients described, might result in very high percentages of Hgb F and lesser amounts of Hgb E. In children under the

age of 2 or 3 when Hgb F is produced physiologically, increased quantities of the fetal pigment would also be expected.[38] Finally, as already pointed out, the absence of Hgb A in this study may be more apparent than real, since small amounts of the normal adult compound, especially in the presence of significant concentrations of Hgb F, may not be detectable by the paper electrophoretic technique employed in this study.

From the genetic standpoint, one other problem is of great interest. In the kinships of patients with thalassemia-Hgb E disease, the parent who does not transmit the hereditary factor for Hgb E has been shown to have hematologic changes compatible with very mild thalassemia. In view of the incidence of what appears to be true Cooley's anemia in the Orient, it is not surprising to find carriers of the thalassemia defect. Numerous reports of Cooley's anemia in non-Mediterranean people suggest that the specific mutation leading to thalassemia arose in a number of independent areas or that the mutation has been widely disseminated from a single focus. One such focus has been postulated in Mongolia, from where the genetic defect may have been carried westward by the Mongol hordes into Europe and the Mediterranean area and southward into China, Thailand, Burma, India, etc.[43] In view of the great difficulties, already alluded to, involved in making a diagnosis of the mild forms of thalassemia, it is doubtful whether the necessary data to evaluate this possibility will ever be at hand.

Another concept which must be given serious consideration is that the genetic defect found in the non-Hgb E parents simulates thalassemia but is not identical with it.[14] Possibly in favor of this idea is the observation that a total of six children were seen in the course of the present study[44] and the one previously reported[1] who had a severe hemolytic process which mimicked Cooley's anemia except for the absence of Hgb F and the presence of inclusion bodies of ill-defined nature, both extremely unusual occurrences for thalassemia major.[14] The parents of these children had hematologic findings which are similar to those of the non-Hgb E parent in thalassemia-Hgb E disease. It may be postulated that the "inclusion" type of hemolytic anemia represents the homozygous form of the thalassemia-like genetic defect and when combined with Hgb E results in thalassemia-Hgb E disease.[14] If this were true, thalassemia-Hgb E disease would be a misnomer. Against this concept, however, is the observation that four patients were encountered in Thailand with an anemia which fulfills all known criteria for Cooley's anemia, two of which were of pure Chinese extraction and two of pure Thai ancestry. In the present state of our knowledge, therefore, it seems best to continue the use of the term thalassemia-Hgb E disease while recognizing the possibility that other hereditary defects may be involved.

The studies on thalassemia in Thailand reported by Minnich and associates[1] described thirty-two patients with this syndrome. Seven of the thirty-two were reinvestigated in the present study. These were Cases 7, 9, 10, 15, 16, 30, and 31, in Table I of the previous report. The first five were shown to be suffering from thalassemia-Hgb E disease (Cases 20, 6, 5, 29, and 24, respectively,

Table II, in this communication) while two (Cases 30 and 31 of previous report) had the "inclusion body" type of Cooley's anemia. Of the remaining twenty-five patients not seen, twenty-two are believed to represent instances of thalassemia-Hgb E disease by virtue of family studies or a review of their clinical or hematologic manifestations. Three of the twenty-two patients have died since the first report of this syndrome (Cases 8, 11, and 29, in report by Minnich and associates) and one (Case 8) was the twin brother of a patient with thalassemia-Hgb E disease (Case 20, Table II this paper). The three remaining patients include two with inclusion body type of hemolytic anemia and one may be an example of Cooley's anemia.

The differential diagnosis of the Hgb E syndromes may at times present difficulties. There are no distinctive changes associated with the Hgb E trait which would aid in making the diagnosis clinically. By contrast, Hgb S trait is easily identified by the sickling phenomenon and the Hgb C trait may be suspected on the basis of the many target cells seen on the stained blood film. Only by utilizing the electrophoretic technique can the diagnosis of Hgb E trait be made. Homozygous Hgb E disease, on the other hand, can be suspected when one encounters a patient with a mild microcytic normochromic anemia in whom target cells are very prominent and the osmotic fragility curve is shifted symmetrically to the right. Only one other syndrome, homozygous Hgb C disease, resembles pure Hgb E disease. Since Hgb C has not yet been found in the Orient, there is little likelihood of confusing the two. Should Hgb E and Hgb C be found in the same area, however, the differential diagnosis would be most difficult, especially since the electrophoretic patterns at pH 8.6 are not too dissimilar. Homozygous Hgb C disease tends to have a somewhat higher percentage of target cells and is less likely to be as microcytic as homozygous Hgb E disease. Splenomegaly is generally more prominent in Hgb C disease than in Hgb E disease. Perhaps most critical, however, will be the demonstration of the hemoglobin patterns at pH 6.5 by means of moving boundary electrophoresis where the two pigments are easily distinguished.

Thalassemia-Hgb E disease may be confused with Cooley's anemia or any of the other thalassemia-hemoglobin disorders. Differential diagnosis will depend almost entirely on the electrophoretic pattern, since the clinical and routine hematologic manifestations are not sufficiently distinctive for practical use. Further study on the incidence of spherocytosis in thalassemia-Hgb E disease may indicate that this morphologic alteration of the erythrocytes is a regular feature of the anemia. Spherocytosis may, then, be a helpful guide in alerting one to the possibility of thalassemia-Hgb E disease.

SUMMARY

The clinical, hematologic, and genetic manifestations of the Hgb E syndromes are described. Hgb E trait, the heterozygous form of Hgb E, is unaccompanied by any disability. Thalassemia-Hgb E disease simulates thalassemia major in almost every respect and results from the mating of a carrier of the gene for thalassemia with an individual who harbors the gene for

Hgb E. Homozygous Hgb E disease is manifest as a mild microcytic, normochromic anemia, characterized by large numbers of target cells. Theoretical considerations relative to the genetic background of thalassemia-Hgb E disease and the thalassemia syndromes are discussed.

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STUDIES ON HEMOGLOBIN E

II. The Incidence of Hemoglobin E in Thailand

Supa Na-Nakorn, M.D., Bangkok, Thailand, Virginia Minnich, M.S., and Amoz I. Chernoff, M.D., St Louis, Mo.

With the Technical Assistance of Suwannee Quagqui-Puag, R.N., and Kusuma Chavalekviraj, R.N.

IN THE preceding communication, the clinical, hematologic and genetic manifestations of thalassemia-hemoglobin E disease have been extensively described.[1] Because thalassemia-Hgb E disease is so frequently encountered in Thailand it seemed of value to study the population of Thailand to determine the prevalence of Hgb E carriers. This communication summarizes the results of electrophoretic studies performed on 1,200 random hemoglobin samples. Hgb E was found in 13.6 per cent of 1,006 individuals of Thai extraction but was not detected in any of 213 racially unmixed Chinese. Further evaluation of the data in relation to racial background, sex, state of health of the individual, and geographic origin of the subject was made. The incidence of malaria could not be correlated with the type of hemoglobin.

MATERIALS AND METHODS

Blood samples were obtained from 1,006 racially mixed and unmixed Thai, 213 pure Chinese, and 11 individuals from 8 other different Asiatic races. Subjects were selected at random, except that an attempt was made to sample both the "normal" and the patient populations. The "normal" group consisted of house staff, medical students, and nurses. The patient group was selected from the outpatient clinic and wards of Siriraj Hospital and from persons seen in other clinics in Thailand.* In order to eliminate weighting of the figures, specimens obtained from families known to harbor one of the Hgb E syndromes are not included in this study.

Three milliliters of venous blood was collected in either potassium-ammonium oxalate mixture or in 5 per cent ethylene diamine tetraacetic acid, disodium salt. Hemoglobin solution was prepared according to the method previously described.[2] Analyses of the hemoglobin types were made by the paper electrophoresis technique modified from the method of Smith and Conley,[3] as described in a previous publication.[4] Fetal hemoglobin determinations were carried out by the alkali denaturation test.[2]

A short history was taken when the blood sample was collected. Information was obtained on nearly all persons as to age, sex, birthplace, race of the four grandparents, and the history of malaria.

From the Department of Medicine, University of Medical Sciences, Siriraj Hospital, Bangkok, Thailand, and the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.

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RESULTS

Results obtained in the survey of the occurrence of Hgb E in the population of Thailand are tabulated in Table I. Of 1,006 pure Thai and racially mixed Thai, Hgb E was found in 137 instances or 13.6 per rent. Of the 137 individuals with Hgb E, 134 were found to have the Hgb trait, with the concentration of Hgb A being greater than that of Hgb E on visual inspection of the paper electrophoresis strips. The hemoglobins of three individuals consisted almost entirely of Hgb E. Two of these had Hgb E plus a slightly increased amount of Hgb F. These three persons probably had homozygous Hgb E disease, although genetic studies were not made to support the diagnosis.

An attempt was made to evaluate the effect of an admixture of non-Thai ancestry on the incidence of Hgb E among the Thai. Racial origin of parents and grandparents was recorded by those obtaining the blood specimens. In many cases, the reliability of such information depended on the cooperation and interest of the subject. At times, Thai ancestry was claimed because of certain socio-economic advantages felt to be associated with such

TABLE I. INCIDENCE OF HGB E IN THE ASIATIC RACES IN THAILAND

RACE	TOTAL NUMBER	NUMBER WITH Hgb E	PER CENT
Racially unmixed and mixed Thai	1,006	137	13.6
Racially unmixed Chinese	213	0	0
Other Asiatic races			
Japanese	1	0	
Vietnamese and Chinese-Vietnamese	2	0	
Lao	1	0	
Mon and Chinese-Mon	3	0	
Indian	2	0	
Lao-Cambodian	1	1	

background. Particularly prominent was the lack of desire on the part of some individuals to admit to Chinese ancestry, especially when they were of pure Chinese extraction. Consequently, the data on racial background are felt to be weighted somewhat in the direction of pure Thai origin. Nevertheless, the conclusion seems inescapable that the incidence of Hgb E is greater in people of unmixed Thai extraction than it is in Thai of mixed ancestry (Table II). In individuals of pure Thai extraction, an incidence of 17.4 per cent was noted; in those with ½ to ⅞ Thai ancestry, 11.0 per cent; and in those with ⅛ to ⅜ Thai heritage, 4.3 per cent. Application of the χ^2 test to the difference between the incidence of Hgb E in persons of pure Thai ancestry and in those only ½ to ⅞ Thai gave a p value of approximately 0.01. Consequently, differences of this magnitude would be expected to occur on a chance basis alone about one time in 100. With the adoption of a p value of 0.05 or less as the level of significance, the greater incidence of Hgb E in pure Thai must be considered of importance. Since the number of individuals comprising the ⅛ to ⅜ group of Thai is small, statistical treatment of these data is not of great moment, although a p value of approximately 0.02 was obtained.

TABLE II. THE INCIDENCE OF HGB E IN RACIALLY MIXED AND UNMIXED THAI

	TOTAL NUMBER	NUMBER WITH Hgb E	PER CENT
Pure Thai	546	95	17.4
½ to ⅞ Thai extraction*	354	39†	11.0
⅛ to ⅜ Thai extraction	51	2	3.9

*328, Thai-Chinese. 26 Thai plus one or more of the following races: Mon, Lao, Cambodian, Vietnamese, Ceylonese, Burmese, Malayan, Filipino, Japanese, Indian, English, Danish, and German.

†33 Thai-Chinese.

No abnormal hemoglobin was found in blood specimens obtained from 213 persons of pure Chinese extraction, 111 of whom were born in China. (Table I) The absence of Hgb E in racially unmixed Chinese is a remarkable finding. However, since the Chinese are of a different race than the Thai[5] their lack of Hgb E is not surprising and would suggest a minimal amount of intermingling of the two groups even during times when war and economic necessity brought them into close contact for long periods. According to Thai historians,[6, 7] the Thai people originated on the Banks of the Yellow River in North China about 7,000 years ago. They were driven in successive waves southward into Yünnan Province and their present homeland by the Chinese. During their years of wandering, however, the Thai maintained their racial integrity and only recently has there occurred appreciable admixture with neighboring groups. Although our present observations lead us to believe that the incidence of Hgb E must be quite low among the Chinese, it will be necessary to analyze many more samples of blood before it can be categorically stated that Hgb E does not occur in this race.

Blood specimens of only a few persons from other Asiatic races were tested. One individual of Lao-Cambodian ancestry was found to have Hgb E in his blood. The remaining were free of the abnormal pigment (Table I).

In a total of nearly 1,500 samples of blood tested in this and other investigations in Thailand no types of hemoglobin other than hemoglobins A, F, and E were found.

Data concerning the incidence of Hgb E in relationship to the sex and state of health of the subjects, i.e., "normals" versus patients, are tabulated in Table III. Fifty-two of 449 males or 11.6 per cent, and 85 of 557 females, or 15.3 per cent had Hgb E as one component of their hemoglobins. The difference between these two figures subjected to the χ^2 test yielded a p value of greater than 0.10. This difference in the incidence of Hgb E between males and females is not statistically significant. On the other hand, 17.1 per cent of the patient group but only 11.2 per cent of the normal group carried the gene for Hgb E. Application of the χ^2 test to these percentages suggests a high degree of significance to the difference ($p < 0.01$). If one analyzes the figures on the incidence of Hgb E among males and females and among normals and patients according to racial background, several interesting relationships become apparent. Sixteen per cent of pure Thai males and 18.7 per cent of pure Thai females have Hgb E, percentages which are not significantly

different ($p > 0.10$). Similarily, the difference in the frequency of Hgb E among males of pure Thai extraction, whether normals or patients, is not of statistical significance (14.8 per cent vs. 17.2 per cent with $P > 0.10$). In the female groups, however, over twice as many of the patient group harbor the gene for Hgb E in comparison with the normal group (27.2 per cent and 13.5 per cent, respectively). The p value of <0.005 for the difference between these two percentages indicates a high degree of significance. Further breakdown of the data does not add any cogent information, since the groups involving subjects of only part Thai ancestry are too small to evaluate. Additional studies in comparing larger numbers of individuals are being carried out in Thailand and will be subjected to similar analyses at a later date.

TABLE III. THE INCIDENCE OF HGB E AMONG MALES AND FEMALES OF "NORMAL" AND PATIENT GROUPS (PURE CHINESE EXCLUDED)

	TOTAL NUMBER	NUMBER WITH Hgb E	PER CENT
Normal males	264	26	9.9
Pure Thai	115	17	14.8
Mixed Thai	149	9	6.1
Patient males	185	26	14.0
Pure Thai	104	18	17.2
Mixed Thai	81	8	10.0
Total	449	52	11.6
Normal females	328	40	12.2
Pure Thai	200	27	13.5
Mixed Thai	128	13	10.0
Patient females	229	45	19.6
Pure Thai	121	33	27.2
Mixed Thai	108	12	11.0
Total	557	85	15.3
Pure Thai males, normal and patient	219	35	16.0
Pure Thai females, normal and patient	321	60	18.7
Normal males and females	592	66	11.2
Patient males and females	414	71	17.1

TABLE IV. INCIDENCE OF HGB E IN DIFFFERENT SECTIONS OF THAILAND (PURE CHINESE EXCLUDED)

SECTION	TOTAL NUMBER	NUMBER WITH Hgb E	PER CENT
Central	554	74	13.3
North	99	8	8.0
Northeast	49	16	32.7
Southeast	48	9	18.7
South	126	16	12.7

The incidence of Hgb E in various parts of Thailand was studied by obtaining information regarding the birthplace of 876 Thai. The greatest number, 554, were from the central area of Thailand, where Bangkok is located (Table IV and Fig. 1). In this group there were 74 instances of Hgb E or 13.3 per cent. Only a small number of blood specimens were obtained from people of other sections of the country, so that the percentages cannot be ade-

quately treated statistically. The highest incidence was in the northeast in which 16 of 49 were found to be positive. Of these 49, 36 individuals claimed to be pure Thai, and 15 of these had Hgb E. Bloods of 8 of 99 persons from the north were positive for Hgb E; 9 of 49 from the southeast; and 16 of 126 from the south.

In view of the recent interest in Africa regarding the possible protective effect of sickle cell hemoglobin on the severity of parasitization by malarial organisms[8-15] it seemed of interest to determine whether carriers of Hgb E

Fig. 1.—Distribution of Hgb E in different sections of Thailand.

were less prone to develop clinical malaria than those without Hgb E. Consequently, 636 individuals were questioned regarding a history of malaria (Table V). Malarial parasites were not searched for and no effort was made to document the diagnosis. Furthermore, since many febrile diseases are assumed to be malaria by the native physician, the true incidence of this disease remains questionable. Five hundred and sixty of the individuals questioned did not have Hgb E. Of this group, two hundred and seventy, or 49.7 per cent, gave a positive history of malaria. Thirty-five of the 76 individuals with Hgb E, or 46.0 per cent, stated that they had experienced an attack of malaria at sometime during their lives. The difference between the two groups is not

statistically significant. While these data are admittedly inadequate, they fail to show that Hgb E offers any protection against the malarial parasite.

TABLE V. THE INCIDENCE OF MALARIA AS DETERMINED BY HISTORY ACCORDING TO THE TYPE OF HEMOGLOBIN (PURE CHINESE EXCLUDED)

	TOTAL NUMBER	PER CENT
Hemoglobin A		
Positive history of malaria	278	49.7
Negative history of malaria	282	50.3
Hemoglobin E		
Positive history of malaria	35	46.1
Negative history of malaria	41	53.9

COMMENT

During the past twenty years, data have been accumulating rapidly on the incidence of various hereditary hemolytic factors. Mass population surveys have been reported on the occurrence of sicklemia, thalassemia minor, and most recently hemoglobin C. A composite survey obtained by many independent investigators and compiled by Margolies[16] has shown that of 22,170 American Negroes tested, 9.0 per cent had sicklemia; and of 18,726 African Negroes, 14.3 per cent had the sickling trait. Considerable variation, however, is encountered in the frequency of the sickle cell trait in different areas of the world, values as high as 45 per cent being noted in some tribes of Central Africa.[17] Silvestroni and Bianco[18] reported that of 30,683 people examined in Italy and Sicily, 2.3 per cent had microcythemia (thalassemia minor), although up to 19.0 per cent of the population at the mouth of the Po River suffered from this genetic abnormality.[19] Hemoglobin C has been found in about 2.0 per cent of the American Negro population[3, 20] and in 14 per cent of West Coast African natives.[21]

The studies reported in this communication establish a remarkably high incidence of Hgb E carriers among the Thai population. In a former report[22] the incidence was reported as 12.5 per cent. However, this figure included persons of pure Chinese extraction. Although the over-all figure of 13.6 per cent is in itself considerable, the data suggest that a higher incidence may be found in individuals of pure Thai extraction. Several aspects of the problems involved, however, need more extensive investigation before any conclusions may be made. First, is the difference in the occurrence of Hgb E between the patient population and the normal, particularly among women, of any real significance? Although statistical evaluation indicates that these differences are of importance, clinical and hematologic studies of the Hgb E trait do not suggest any morbidity related to the heterozygous state for Hgb E. No data were kept on the nature of the diseases associated with the Hgb E trait, but such information might be of extreme importance in the future evaluation of this problem. Second, it would be of interest to know whether the high incidence of Hgb E in one part of the country indicates a focus of anthropologic

importance in the northeast area. The number of subjects studied has been small and the sampling of the population is weighted by the high percentage of pure-blooded Thai. Nonetheless, the suggestive nature of the results makes it imperative to investigate further the incidence of Hgb E in various parts of the country.

A third problem of the greatest interest, left unanswered by the present studies, relates to the total expression of Hgb E among people of southeast Asia. Information emanating from various laboratories indicates that Hgb E is present in Indonesians in 3.6 per cent of 500 subjects[23] and in Ceylonese (2 out of 9).[24] Cases of thalassemia reported from Burma[25] are similar to our patients with thalassemia-Hgb E disease so that Hgb E may well exist in Thailand's immediate western neighbor. Finally, Hgb E has been detected in a Persian woman residing in the United States who is the mother of a child with thalassemia-Hgb E disease.[26] The significance of Hgb E as an anthropologic tool can only be surmised at present, but the rather limited distribution of this genetic defect would suggest that the detection of Hgb E may prove extremely useful in anthropologic studies in southern Asia. The apparent absence of Hgb E in Chinese, furthermore, enhances the value of Hgb E detection as a tool in the investigation of population origins and migrations.

Three individuals in whose blood Hgb E was predominant were encountered in the present survey. In all probability, they represent instances of homozygous Hgb E disease. With an incidence of heterozygotes of about 13.5 per cent, one would expect 4 individuals with homozygous Hgb E disease in a random population sample of about 1,000 people, provided a single pair of genes controls the type of hemoglobin formed.* Data, presented in the previous paper,[1] support the concept that Hgb E is an allelomorph of Hgbs A, S, and C and may therefore be expected to occur in the homozygous as well as the heterozygous form.

No study has been carried out on the incidence of thalassemia minor in Thailand, even though that information would be invaluable in view of the frequency with which one encounters thalassemia-Hgb E disease. The difficulties encountered in identifying the lesser forms of thalassemia, discussed in the previous report, make this a most complicated task. Should the defect of thalassemia be more critically defined, a survey of the Thai population will undoubtedly reveal a high incidence for this abnormality. Since thalassemia-Hgb E disease is the cause of so much illness in Thailand, it may then be possible to avoid its occurrence by the judicious use of marriage counseling in patients harboring these genetic defects.

*The probability of an AE and an AE mating is 185 in 10,000 marriages. 13.6/100 × 13.6/100 = 185/10,000. Twenty-five per cent of the children resulting from AE and AE marriages will be EE. The number of homozygous E children, therefore, will be 4.6 per 1,000 Thai population.

$$\frac{1.85}{\frac{4}{100}} = \frac{.46}{100} \text{ or } 4.6/1{,}000$$

SUMMARY AND CONCLUSIONS

1. Paper electrophoretic analyses of the hemoglobin types of 1,006 individuals of Thai ancestry revealed 137 instances of Hgb E, or 13.6 per cent. Three subjects with what is believed to be homozygous Hgb E disease were detected. No abnormal hemoglobin was found in blood specimens obtained from 213 racially unmixed Chinese. Hgb E was found in one individual of Lao-Cambodian extraction.

2. The incidence of Hgb E in Thai increases in a significant manner as the degree of racial purity increases.

3. No significant differences were observed in the incidence of Hgb E in males and females. A higher rate of occurrence of Hgb E occurred among patients than among normal controls but only among the Thai women did these differences appear to be significant.

4. Hgb E apparently offers no noticeable protection against malaria.

5. The significance of these observations is discussed.

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LABORATORY METHODS

THE ISOLATION OF HISTOPLASMA CAPSULATUM FROM SPUTUM AS A DIAGNOSTIC PROCEDURE

GENTRY W. MOFFIT, M.P.H., BERNARD H. MORGAN, B.S., AND
GEORGE M. CAMERON, PH.D.
NASHVILLE, TENN.

INTRODUCTION

THE difficulties encountered in the attempt to isolate pathogenic fungi from sputum by direct culture methods have been recognized by many workers.[5] Usually it has been true that the culture medium is overgrown by either bacteria or saprophytic fungi long before the slow-growing pathogenic fungi have time to appear. More recently the use of cycloheximide[6] (Acti-dione) in the medium, as an inhibitant for saprophytic fungi, has helped greatly in direct cultures.

Nevertheless, a satisfactory method for concentrating the fungus elements in a sputum specimen for culturing purposes has not been described. Ajello[2] found the most commonly used tubercle bacillus concentration procedures unsuitable for use with fungi causing pulmonary mycoses.

Emmons[3, 4] and Ajello[1] used the inoculation of white Swiss mice as a means of isolating *Histoplasma capsulatum* from material highly contaminated with bacteria and saprophytic fungi, such as soil. The Division of Laboratories, Tennessee Department of Public Health, has adapted this method to the isolation of *Histoplasma capsulatum* from sputum.

One of the authors in previous work (unpublished) demonstrated that the concentration of the tissue phase of *Histoplasma capsulatum* in sputum can be effected by diluting the specimen with an equal volume of physiological saline, shaking in a paint conditioner, and applying centrifugation. A concentrate prepared in this manner with antibiotics added proved to be suitable for inoculation into mice. Since the contaminants, especially saprophytic fungi present in the sputum, were also concentrated by this procedure, the sediment was less suitable for direct culture than the untreated specimen. Here again the use of Acti-dione in the medium might prove quite advantageous. For the experiments reported in this paper, Acti-dione was not used in the isolating medium.

From the Division of Laboratories, Tennessee Department of Public Health.

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A report on results obtained from culturing 193 sputum specimens for *Histoplasma capsulatum,* with use of both direct culture and mouse inoculation methods, is herein presented. The sputum specimens used in this study were routine specimens submitted to the laboratory with a request for fungus studies.

MATERIALS AND METHODS

Direct cultures were made from the untreated portion of the sputum specimens, after removal of approximately 3 ml. for concentration. The direct culture inoculations were made from each sputum specimen on three Sabouraud's glucose agar slants, one of which contained antibiotics, 20 units of penicillin, and 40 units of streptomycin per milliliter.

The approximately 3 ml. portion of each specimen used for concentration was placed in a sterile 16 × 125 mm. screw-cap culture tube, and to this an equal volume of sterile physiologic saline was added. The tube and contents were then shaken for 10 minutes in a paint conditioner and subsequently centrifuged at 2,500 r.p.m. for 15 minutes. When a sputum was highly mucoid, a tube containing a few small glass beads was used in this procedure as an aid in emulsifying the sputum. A sterile blunt pipette equipped with a 2 ml. bulb was used to remove the excess supernatant fluid, and slightly more than 1.5 ml. of the material was left in the tube. In removing the excess supernatant fluid care was taken not to disturb the sediment and not to remove the surface layer of the liquid so that both high and low specific gravity particles would be included in the inoculum. Approximately 10,000 units of penicillin and 10,000 units of streptomycin were added to the sediment and allowed to stand at room temperature for 20 minutes.

Mouse inoculations from each sputum specimen were performed by intraperitoneal injection into each of three white Swiss mice with use of 0.5 ml. amounts of this concentrated sputum sediment containing streptomycin and penicillin. The mice used were from 4 to 6 weeks of age.

Routinely, the mice were sacrificed at the end of 28 days, although in certain instances mice were sacrificed and isolations accomplished at the end of 2 weeks, and in one case at the end of eight weeks. The spleen and liver were minced with sterile scissors and a small amount of each organ was inoculated onto three Sabouraud's glucose agar slants, one of which contained 20 units of penicillin and 40 units of streptomycin per milliliter.

All cultures made directly from the sputum specimens and from the mouse tissues were incubated at 25° C. for a period of 4 weeks. Weekly examinations of the cultures were made.

The identification of *Histoplasma capsulatum,* in all cases, was made by demonstration, microscopically, of the characteristic morphology of the tuberculate spore. In a number of cases the yeast form was also demonstrated. All isolations save one are available in this laboratory for further study.

RESULTS

Histoplasma capsulation was isolated, from 12 of the 193 sputum specimens studied, and presented in this report.

As can be seen in Table I, 12 or 6.2 per cent were positive by mouse inoculation, while only 4 or 2.1 per cent were positive by direct culture.

There were no specimens positive for *Histoplasma capsulatum* by direct culture that were negative by mouse inoculation. However, 8 specimens were positive by mouse inoculation which were negative by direct culture.

Almost without exception, direct cultures negative for *Histoplasma capsulatum* which were prepared from specimens later found to be positive by mouse inoculation were overgrown with contaminants.

TABLE I. TOTAL AND PER CENT ISOLATIONS OF HISTOPLASMA CAPSULATUM FROM 193 SPUTUM SPECIMENS SHOWING ALSO NUMBER AND PER CENT POSITIVE BY DIRECT CULTURE AND BY MOUSE INOCULATION

NUMBER SPUTUM SPEC.	TOTAL POSITIVE		POSITIVE MOUSE INOCULATION		POSITIVE DIRECT CULTURE		POS. MOUSE INOC. NEG. DIRECT CUL.		POS. DIRECT CUL. NEG. MOUSE INOCULATION	
	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
193	12	6.2	12	6.2	4	2.1	8	4.1	0	0

DISCUSSION

Clinical histories of the patients involved in this study were supportive of the laboratory findings.

Of the nine cases involved, x-ray studies indicated lung cavities in seven and infiltration in the right lung in an eighth. The ninth patient died but the cause of death remained obscure, and the autopsy showed a diffuse granulomatous disease.

From two cases represented by four specimens of sputum, *Histoplasma capsulatum* was also demonstrated in post-mortem examination. One of these cases and two others gave positive complement fixation tests with *Histoplasma capsulatum* antigen.

Two patients found to have *Histoplasma capsulatum* in the sputum by the methods used were also shown by culture to have *Mycobacterium tuberculosis* var. *hominis* in the sputum. One of these gave both a positive histoplasmin skin test and a positive complement fixation test for histoplasmosis. Results of these tests were not available for the second patient. One or more sputum specimens cultured for the tubercle bacillus from each of the remaining seven patients were negative. All microscopic examinations of sputum specimens from these seven patients were also negative for acid-fast organisms.

Since a majority of the mice were sacrificed within four weeks and none later than eight weeks following inoculation, the post-mortem examination frequently revealed no significant lesions, spleen enlargement, or other gross pathology. In some instances where isolations of *Histoplasma capsulatum* were made, spleen enlargement was evident; but frequently isolations were made from mice with spleens showing no apparent enlargement. The absence of spleen enlargement in the latter cases was possibly due to the fact that these mice received a light infective dose, plus the relatively short incubation period.

Spleen enlargement was noted in some sacrificed mice from which no isolations of *Histoplasma capsulatum* were made. This was thought to be due to an initial bacterial infection from which the mouse had not completely recovered when sacrificed.

During this study no uninoculated mice were used as controls. However, in view of the large number of inoculated mice found negative for *Histoplasma capsulatum* combined with the supporting clinical picture of the patients with sputum found positive for *Histoplasma capsulatum,* it was felt that the mouse colony of this laboratory could be considered free of natural infection with this fungus.

The data herein presented indicate that the mouse inoculation method for isolating *Histoplasma capsulatum* from sputum offers advantages over the direct culture method used in this work.

A better control of interfering contaminants, especially the saprophytic fungi, was obtained by the mouse inoculation method.

Use of a greater amount of inoculum gives mouse inoculation an advantage over direct culture methods.

Further comparative studies are now being made using Acti-dione in one tube of the culture medium for direct inoculation and also in a tube of medium inoculated from the concentrated sputum. This procedure gives promise of more effective culture isolation.

SUMMARY

Results obtained in examining 193 sputum specimens for *Histoplasma capsulatum* with use of the direct culture and mouse inoculation methods have been presented. Twelve or 6.2 per cent of the 193 specimens were positive for *Histoplasma capsulatum* by the mouse inoculation method, while only 4 or 2.1 per cent were positive by direct culture.

A method for the preparation of sputum to be inoculated into mice has been described.

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